67

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(54) Title: HYPOXIA INDUCING FACTORS AND USES THEREOF FOR INDUCING ANGIOGENESIS AND IMPROVING MUSCULAR FUNCTIONS

hHIF-3a (2223 bps)
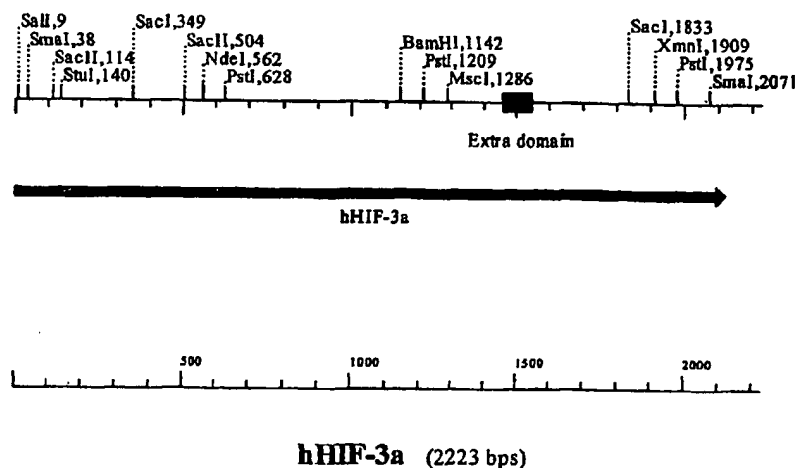
(57) Abstract: This invention provides HIF-3α nucleic acid and sequences. Also provided are methods for using HIF-3α nucleic acids, proteins, fragments, antibodies probes, and cells, to characterize HIF-3α, modulate HIF-3α cellular levels, induce angiogenesis, improve muscular functions, and treat coronary and cardiac diseases in mammals.

WO 02/094862 A2

HYPOXIA INDUCING FACTORS AND USES THEREOF FOR INDUCING ANGIOGENESIS AND IMPROVING MUSCULAR FUNCTIONS

RELATED APPLICATIONS

5   This application claims priority of United States Provisional Applications 60/292,630 filed May 23, 2001, and 60/354,529 filed February 08, 2002.

BACKGROUND OF THE INVENTION

a)   Field of the invention

10.   The present invention is concerned with a human protein called "HIF-3α" that is a $\underline{H}$ypoxia $\underline{I}$nducible $\underline{F}$actor-3α and more particularly to the use of HIF-3α nucleic acids, proteins, fragments, antibodies, probes, and cells, to characterize HIF-3α, and modulate its cellular levels.

The present invention is also concerned with the use of nucleotide 15   sequences encoding for proteins from the hypoxia inducible factors family for inducing VEGF expression, for inducing angiogenesis and for improving muscular functions, and more particularly, for treating coronary and cardiac diseases in mammals.

20   b)   Brief description of the prior art

Chronic ischemic heart disease is a worldwide health problem of major proportions. According to the American Heart Association, 61 800 000 Americans have at least one type of cardiovascular disease. In particular, coronary heart disease (CHD) cause myocardial infarction (MI) for 7 500 000 American patients 25   and congestive heart failure (CHF) for 4 800 000 American patients. Almost 450 000 deaths in the United States alone were deemed to derive from CHD.

Current CHD treatments include medication, percutaneous transluminal coronary angioplasty and coronary artery bypass surgery. These procedures are quite successful to increase blood flow in the myocardium thus reducing ischemia 30   and ameliorating the condition of the patient. However, due to the progressive nature of CHD, the beneficial effects of these procedures are not permanent and new obstructions can occur. Patients that live longer through effective

2

cardiovascular interventions eventually run out of treatment options. Also an important patient population is still refractory to these treatments due to diffuse athereosclerotic diseases and/or small caliber arteries.

Severe and chronic ischemia can cause MI which is an irreversible scarring
5   of the myocardium. This scarring reduces heart contractility and elasticity and consequently the pumping function, which can then lead to CHF. Treatments available to CHF patients target kidney function and peripheral vasculature to reduce the symptoms but none are treating the scar or increasing pump function of the heart. A very promising approach for reducing the scar and improving heart
10 function is named cellular cardiomyoplasty (CCM). It consists in the injection of cells in the scar, replacing the fibrotic scar by healthy tissue and increasing elasticity (see US patents No. 5,130,141; No. 5,602,301, No. 6,099,832 and No. 6,110,459).

Another emerging treatment for CHF patients is therapeutic angiogenesis.
15 Angiogenesis is defined as blood vessel sprouting and proliferation from pre-existing vasculature. The net result is a higher capillary density and better blood perfusion. For instance, US patent No. 5,792,453 disclose a method for promoting coronary collateral vessel development by delivering an adenovirus vector with a transgene encoding for an angiogenic protein. Although stimulation of
20 angiogenesis can improve function of ischemic myocardium, it will have no effect on scar tissue because no viable cells will benefit from the improved perfusion.

Many growth factors are currently used to induce angiogenesis, including Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), but none of these factors has the property to stimulate every step of angiogenesis
25 (basal membrane disruption, endothelial cell proliferation, migration and differentiation followed by periendothelial cells recruitment). Since it is known that cell hypoxia can naturally induce a strong angiogenesis, the use of regulators of hypoxia could stimulate the synthesis of one or many angiogenic factors at once, thereby resulting in a more structured and stronger angiogenesis than with
30 individual factors.

Hypoxia Inducible Factors (HIFs) are heterodimeric transcription factors that regulate a number of adaptive responses to low oxygen tension. They are

composed of alpha- and beta-subunits that belong to the basic helix-loop-helix-PAS (bHLH-PAS) superfamily. Members of this family include HIF-1α (also known as MOP1; see Wang *et al.*, *Proc. Natl. Aca. Sci. USA* (1995) 92:5510-5514; and U.S patents No. 5,882,314; 6,020,462 and 6,124,131), HIF-2α (also known as
5   Endothelial PAS 1 (EPAS1), MOP2, HIF-related factor (HRF) and HLF (HIF-like factor), see Tian *et al.*, *Genes & Dev.* (1996) 11:72-82; and U.S patent No. 5,695,963).

Another member of the HIF family has been discovered recently, namely HIF-3α. The cloning of HIF-3α has been described in mice (Gu *et al.*, *Gene*
10 *Expression* (1998) 7:205-213; and in International PCT application WO 99/28264) and in rat (Kietzmann *et al.*, *Biochem J.* (2001), 354 (Pt3):531-537). A partial cDNA sequence of human HIF-3α has been published in 1999 (GenBank™ accession No. AF079154), and a full length sequence of a human HIF-3α isoform, different from the one of the present invention, was published in October 2001 by
15 Hara *et al.* (*Biochem. Biophys. Res. Comm.* (2001), Oct 5; 287:808-813).

HIFs are highly labile in normal conditions, but are stabilized in response to low oxygen tension. This stabilization allows them to bind to *cis* DNA elements of target genes, and stimulate transcription of hypoxia induced genes that help cell survival in low oxygen conditions. These target genes are implicated in processes
20 such as anaerobic metabolism (glucose transporters and glycolytic enzymes), vasodilatation (inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1)), increased breathing (tyrosine hydroxylase), erythropoiesis (erythropoietin) and angiogenesis (VEGF). Gene activation by HIF-1α or HIF-2α was demonstrated by co-transfection assays, in which a reporter gene is activated by
25 the co-transfected HIF factor (Tian *et al.*, *Genes & Dev.* (1996) **11**: 72-82; Jiang *et al.*, *J. Biol. Chem.* (1995) **272**: 19253-19260). The role of HIF-2α in VEGF activation was also demonstrated in renal cell carcinoma (Xia *et al.*, Cancer (2001), **91**:1429-1436). In animal models, strong angiogenesis was reported following gene transfer of a hybrid HIF-1α/VP16 DNA construct (Vincent *et al.*,
30 *Circulation* (2000) **102**: 2255-2261). However, prior to the present invention, it has never been demonstrated or suggested that HIF-2α or HIF-3α could induce the expression of angiogenesis-related gene(s) in mammalian muscular cells, nor that

4

they could induce angiogenesis in these cells. It was also unknown that expression of HIF-1α, HIF-2α or HIF-3α in ischemic muscular tissue resulted in an increased metabolic activity of this tissue, indicating improved function.

Given that HIFs seem to represent ideal factors for VEGF activation and/or for induce angiogenesis, there is thus a need to identify a novel member of the HIF family. There is more particularly a need for a human HIF-3α protein and a nucleic acid encoding the same.

Also, it would be highly desirable to be provided with methods, compositions and cells for inducing angiogenesis and for improving muscular functions.

The present invention fulfils these needs and also other needs as it will be apparent to those skilled in the art upon reading the following specification.

SUMMARY OF THE INVENTION

The present inventors have discovered a novel member of the human Hypoxia Inducible Factors (HIFs) HIF-3α. The present inventors have also discovered uses for human HIF-3α proteins, fragments, nucleic acids, and antibodies for modulating HIF-3α cellular levels, for inducing VEGF expression in a mammalian cell, and for inducing angiogenesis in a mammalian tissue.

In general, the invention features an isolated or purified nucleic acid molecule, such as genomic, cDNA, antisense, DNA, RNA or a synthetic nucleic acid molecule that encodes or corresponds to a human HIF-3α polypeptide.

According to a first aspect, the invention features isolated or purified nucleic acid molecules, polynucleotides, polypeptides, human HIF-3α proteins and fragment thereof. Preferred nucleic acid molecules consist of a cDNA.

In a first embodiment, the isolated or purified nucleic acid molecule encodes a human protein that has the biological activity of a human HIF-3α polypeptide.

According to a specific embodiment, the nucleic acid of the invention comprises a sequence selected from the group consisting of :

a) sequences provided in SEQ ID NO: 1 or 3;

b) complements of the sequences provided in SEQ ID NO: 1 or 3;

c) sequences consisting of at least 20 contiguous residues of a sequence provided in SEQ ID NO: 1 or 3;

d) sequences that hybridize to a sequence provided in SEQ ID NO: 1 or 3, under moderately or strong stringent conditions;

5   e) sequences having at least 75% identity to a sequence of SEQ ID NO: 1 or 3; and

f) degenerate variants of a sequence provided in SEQ ID NO: 1 or 3.

More preferably, the nucleic acid molecule of the invention comprises a sequence selected from the group consisting of:

10   a) a nucleotide sequence having at least 75%, 80%, 85%, 90%, 95% or 97% nucleotide sequence identity with SEQ ID NO: 1; and

b) a nucleotide sequence having at least 75%, 80%, 85%, 90%, 95% or 97% nucleotide sequence identity with a nucleic acid encoding an amino acid sequence of SEQ ID NO:2.

15   Even more preferably, the nucleic acid molecule comprises a sequence substantially the same or having 100% identity with SEQ ID NO: 1 or a sequence substantially the same or having 100% identity with nucleic acids encoding an amino acid sequence of SEQ ID NO: 2. Most preferred nucleic acid molecules are those comprising part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, and/or

20   those comprising part or all of nucleic acids encoding a polypeptide with amino acids 475 to 515 of SEQ ID NO: 2.

According to another specific embodiment, the isolated or purified nucleic acid molecule comprises a sequence encoding a human HIF-3α polypeptide or degenerate variants thereof, the human HIF-3α polypeptide or degenerate variant

25   comprising part or all of amino acids 475 to 515 of SEQ ID NO: 2. Preferably, the purified nucleic acid molecule comprises part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1. In an even more specific aspect, the invention features an isolated or purified human nucleic acid molecule comprising a polynucleotide having the SEQ ID NO: 1, or degenerate variants thereof, and encoding a human

30   HIF-3α polypeptide. Preferably, the nucleic acid is a cDNA and it encodes the amino acid sequence of SEQ ID NO: 2 or a fragment thereof.

6

In a related aspect, the invention features an isolated or purified nucleic acid molecule which hybridizes under low, preferably moderate, and even more preferably high, stringency conditions to any of the nucleic acid molecules defined hereinbefore. More preferably, such nucleic acid molecules hybridizes under moderate or high stringency conditions with part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, or with part or all of a complementary sequence thereof.

The invention also features substantially pure human polypeptides and proteins that are encoded by any of the above mentioned nucleic acids. In one embodiment, the protein has the biological activity of a human HIF-3α polypeptide. Preferred biological activity comprises induction of VEGF expression, thereby promoting angiogenesis.

In another embodiment, the invention aims at an isolated or purified polypeptide comprising an amino acid sequence selected from the group consisting of:

a) sequences having at least 80% identity to SEQ ID NO: 2;

b) sequences having at least 85% homology to SEQ ID NO: 2;

c) sequence provided in SEQ ID NO: 2;

d) sequences having at least 80% identity to amino acid sequences encoded by an open reading frame having SEQ ID NO: 3; and

e) sequences having at least 85% sequence homology to amino acid sequences encoded by an open reading frame having SEQ ID NO: 3.

More preferably, the polypeptide comprises an amino acid sequence selected from the group consisting of:

a) sequences substantially the same as SEQ ID NO: 2; and

b) sequences substantially the same as amino acid sequences encoded by an open reading frame having SEQ ID NO: 3.

In an even more specific aspect, the invention features a substantially pure human HIF-3α polypeptide or a fragment thereof, comprising part or all of amino acids 475 to 515 of SEQ ID NO: 2, or degenerate variants thereof. Even more preferably, the polypeptide comprises an amino acid sequence 100% identical to SEQ ID NO: 2.

The present invention also features protein fragments derived from any of the above mentioned protein or polypeptides. Similarly, the invention further encompasses polypeptides fragment comprising an amino acid sequence encoded by a nucleotide sequence comprising at least 24 sequential nucleic acids

5 of SEQ ID NO:1 (hHIF-3α).

The present invention further features an antisense nucleic acid and a pharmaceutical composition comprising the same. Preferably, the antisense hybridizes under high stringency conditions to a genomic sequence or to a mRNA so that it reduces human HIF-3α cellular levels of expression. According to a first

10 embodiment, the human HIF-3α polypeptide comprises amino acids 475 to 515 of SEQ ID NO: 2. More preferably, the human HIF-3α polypeptide is encoded by an open reading frame having SEQ ID NO: 3. According to a specific embodiment, the antisense hybridizes under high stringency condition with part or all of SEQ ID NO: 1, or with part or all of a complementary sequence thereof. More preferably,

15 the antisense hybridizes under high stringency conditions with part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, with part or all of nucleic acids 2116 to 2223 of SEQ ID NO: 1, or with part or all of complementary sequences thereof.

The present invention further relates to a pharmaceutical composition comprising: (1) at least one element selected from the group consisting of: (i) a

20 nucleic acid molecule encoding a human HIF-3α polypeptide; (ii) a human HIF-3α polypeptide; (iii) an antisense nucleic acid that reduces a human HIF-3α polypeptide levels of expression; and (iv) an isolated or purified antibody that specifically binds to a HIF-3α polypeptide; and (2) a pharmaceutically acceptable carrier or diluent.

25 According to another aspect, the invention features a nucleotide probe comprising a sequence of at least 15, 20, 25, 30, 40, 50, 75 or 100 sequential nucleotides of SEQ ID NO: 1 or of a sequence complementary to SEQ ID NO:1. Preferably, the probe comprises part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, part or all of nucleic acids 2116 to 2223 of SEQ ID NO: 1, part or all of

30 nucleic acids encoding amino acids 475 to 515 of SEQ ID NO: 2, or part or all of a complementary sequence thereof. The invention also encompasses a substantially pure nucleic acid that hybridizes under low, preferably moderate, and more

8

preferably under high stringency conditions to a probe of at least 20, 25, 30, 40, 50, 75 or 100 nucleotides in length that is derived from SEQ ID NO:1.

According to another aspect, the invention features a purified antibody. In a preferred embodiment, the antibody is a monoclonal or a polyclonal antibody that
5   specifically binds to a purified mammalian HIF-3α polypeptide. Preferably, the antibody specifically binds to a HIF-3α polypeptide substantially the same as SEQ ID NO:2. More preferably, the antibody specifically binds to a HIF-3α polypeptide comprising part or all of amino acids 475 to 515 of SEQ ID NO:2 and even more preferably, the antibody specifically binds to part or all of amino acids 475 to 515
10   of SEQ ID NO:2.

In another aspect, the present invention further features a method for inducing VEGF expression in a mammalian cell. The method comprises introducing and expressing in the cell a nucleic acid sequence encoding polypeptide having the biological activity of a human HIF-3α polypeptide. In a
15   preferred embodiment, the cell consists of a cardiac cell located in the heart of a living mammal, and expression of the polypeptide induces angiogenesis in cardiac tissue of the mammal. In another embodiment, the cell consists of a muscular cell located in muscular tissue of a living mammal, and expression of the polypeptide induces angiogenesis in the muscular tissue of the mammal. In another
20   embodiment, the mammalian cell is a skeletal muscular cell thereby providing a HIF-3α expressing-skeletal muscular cell, and the method further comprises the step of transplanting a plurality of the HIF-3α expressing-skeletal muscular cells in a cardiac tissue of a compatible mammalian recipient.

Furthermore, the present invention features a method for inducing
25   angiogenesis in a mammalian tissue having a plurality of cells, the method comprising the step of introducing and expressing in at least some of these cells a nucleic acid sequence encoding a polypeptide having the biological activity of a human HIF-3α polypeptide.

The present invention also features a method for modulating tumoral cell
30   survival or for eliminating a tumoral cell in a mammal, comprising the step of reducing cellular expression levels of a HIF-3α polypeptide. According to a

preferred embodiment, the mammal consists of a human, and a human HIF-3α antisense is introduced into the tumoral cell.

The present invention further features a method for determining the amount of a human HIF-3α polypeptide or a human HIF-3α nucleic acid in a biological

5   sample, comprising the step of contacting the sample with an antibody or with a probe as defined previously.

According to a further aspect, the invention features a method of evaluating malignancy of a tumor in a human subject, comprising the step of measuring the amount of a HIF-3α polypeptide or of a HIF-3α nucleic acid in a tumoral cell from

10   the subject, the amount being indicative of a degree of malignancy for the tumor.

In another related aspect, the invention features a kit for determining the amount of a HIF-3α polypeptide in a sample, the kit comprising an antibody or a probe as defined previously, and at least one element selected from the group consisting of instructions for using the kit, reaction buffer(s), and enzyme(s).

15   The nucleic acids of the invention may be incorporated into a vector and or a cell (such as a mammalian, yeast, nematode or bacterial cell). The nucleic acids may also be incorporated into a transgenic animal or embryo thereof. Therefore, the present invention features cloning or expression vectors, and hosts (such as transformed or transfected cells, transgenic animals) that contain any of the

20   nucleic acids of the invention and more particularly those encoding a HIF-3α protein, polypeptide or fragment, and those capable of directing expression of a HIF-3α protein, polypeptide or fragment in a vector-containing cell.

In a related aspect, the invention features a method for producing a human HIF-3α polypeptide comprising:

25   -   providing a cell transformed with a nucleic acid sequence encoding a human HIF-3α polypeptide positioned for expression in this cell;

-   culturing the transformed cell under conditions suitable for expressing the nucleic acid; and

-   producing the hHIF-3α polypeptide.

30   One of the greatest advantages of the present invention is that it provides nucleic acid molecules, proteins, polypeptides, antibodies, probes, and cells that

can be used for characterizing HIF-3α, modulate its cellular levels, and promotes angiogenesis.

Other objects and advantages of the present invention will be apparent upon reading the following non-restrictive description of the preferred
5   embodiments thereof and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schema illustrating the human HIF-3α gene organization.

Figure 2A is a bar graph illustrating the expression of VEGF in HEK293
10   cells transfected with HIF-3α constructs.

Figure 2B is a bar graph illustrating the expression of VEGF in Hep3B cells transfected with HIF-3α constructs

Figure 3A is a bar graph illustrating the expression of VEGF in HEK293 cells transfected with HIF-2α constructs.

15   **Figure 3B** is a bar graph illustrating the expression of VEGF in Hep3B cells transfected with HIF-2α constructs.

Figure 3C is a bar graph illustrating the expression of VEGF in human skeletal muscle cells transduced with HIF-2α constructs.

Figure 4 is a schema showing a possible process of angiogenesis and the
20   probable role therein of some angiogenesis-related genes.

Figure 5A is a photograph of HIF-2α modified hSkMC implanted subcutaneously in mice showing differentiation in myotubes and an organized vasculature around and in between muscle fiber (arrows point to vessels).

25   **Figure 5B** is a bar graph that shows the angiogenic activity of HIF constructs modified hSkMC.

Figure 6A is a graphic showing the metabolic activity in an ischemic rat heart muscle treated with Ad.HIF-2α.

Figure 6B is a bar graph that shows the measured blood vessel density in
30   the infarcted area treated with myocardial HIF-2α gene transfer in rats.

DETAILED DESCRIPTION OF THE INVENTION

A) D finitions

Throughout the text, the word "kilobase" is generally abbreviated as "kb", · the words "deoxyribonucleic acid" as "DNA", the words "ribonucleic acid" as 5   "RNA", the words "complementary DNA" as "cDNA", the words "polymerase chain reaction"· as "PCR", and the words "reverse transcription" as "RT". Nucleotide sequences are written in the 5' to 3' orientation unless stated otherwise.

In order to provide an even clearer and more consistent understanding of the specification and the claims, including the scope given herein to such terms, 10   the following definitions are provided:

Antisense: as used herein in reference to nucleic acids, is meant a nucleic acid sequence, regardless of length, that is complementary to the coding strand of a gene.

Expression: refers to the process by which gene encoded information is 15   converted into the structures present and operating in the cell. In the case of cDNAs, cDNA fragments and genomic DNA fragments, the transcribed nucleic acid is subsequently translated into a peptide or a protein in order to carry out its function if any. By "**positioned for expression**" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation 20   of the sequence (i.e., facilitates the production of, e.g., a HIF-3α polypeptide, a recombinant protein or a RNA molecule).

Fragment: Refers to a section of a molecule, such as a protein, a polypeptide or a nucleic acid, and is meant to refer to any portion of the amino acid or nucleotide sequence.

25   **Host**: A cell, tissue, organ or organism capable of providing cellular components for allowing the expression of an exogenous nucleic acid embedded into a vector or a viral genome, and for allowing the production of viral particles encoded by such vector or viral genome. This term is intended to also include hosts which have been modified in order to accomplish these functions. Bacteria, 30   fungi, animal (cells, tissues, or organisms) and plant (cells, tissues, or organisms) are examples of a host.

12

Isolat d or **Purified** or **Substantially pure**: Means altered "by the hand of man" from its natural state, i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a protein/peptide naturally present in a living organism is not "isolated", the same

5   polynucleotide separated from the coexisting materials of its natural state, obtained by cloning, amplification and/or chemical synthesis is "isolated" as the term is employed herein. Moreover, a polynucleotide or a protein/peptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism.

10 **Nucleic acid**: Any DNA, RNA sequence or molecule having one nucleotide or more, including nucleotide sequences encoding a complete gene. The term is intended to encompass all nucleic acids whether occurring naturally or non-naturally in a particular cell, tissue or organism. This includes DNA and fragments thereof, RNA and fragments thereof, cDNAs and fragments thereof, expressed

15 sequence tags, artificial sequences including randomized artificial sequences.

Open reading frame ("ORF"): The portion of a cDNA that is translated into a protein. Typically, an open reading frame starts with an initiator ATG codon and ends with a termination codon (TAA, TAG or TGA).

Polypeptide: means any chain of more than two amino acids, regardless of

20 post-translational modification such as glycosylation or phosphorylation.

Percent identity and **Percent similarity**: used herein in comparisons or nucleic acid and/or among amino acid sequences. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer

25 Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Owl 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid,

30 glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

HIF-3α nucleic acid: means any nucleic acid (see above) encoding a mammalian polypeptide that has the biological activity of activating, in an hypoxia inducible fashion, target genes such as VEGF and having at least 75%, 77%, 80%, 85%, 90%, 95%, 97% or 100% identity or homology to the amino acid

5 sequence shown in SEQ. ID. NO: 2. Even more preferably, the mammalian HIF-3α polypeptide comprises amino acids having at least 75%, 77%, 80%, 85%, 90%, 95%, 97% or 100% identity or homology to amino acids 475 to 515 of SEQ ID NO: 2. When referring to a human HIF-3α nucleic acid, the nucleic acid encoding SEQ. ID. NO: 2 is more particularly concerned. **HIF-3α protein** or

10 **HIF-3α polypeptide**: means a protein, a polypeptide, or a fragment thereof, encoded by a HIF3α nucleic acid as described above.

Specifically binds: means an antibody that recognizes and binds a protein or polypeptide but that does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, that naturally includes protein.

15 **Substantially the same**: refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein. With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the

20 same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function of the protein.

25 **Substantially pure polypeptide**: means a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a HIF-3α polypeptide that is at least

30 75%, 80%, or 85%, more preferably at least 90%, 95% or 97% and most preferably at least 99%, by weight, pure. A substantially pure HIF-3α polypeptide

may be obtained, for example, by extraction from a natural source (including but not limited to lung cells, kidney cells, heart cells or any other cell expressing HIF-3α) by expression of a recombinant nucleic acid encoding a HIF-3α polypeptide, or by chemically synthesizing the protein. Purity can be

5   measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from

10   which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes. By "**substantially pure DNA**" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is

15   derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It

20   also includes a recombinant DNA which is part of a hybrid gene encoding an additional polypeptide sequence.

     Transformed or **Transfected** or **Transduced** or **Transgenic cell**: refers to a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a

25   HIF-3α polypeptide. By "transformation" is meant any method for introducing foreign molecules into a cell. Lipofection, calcium phosphate precipitation, retroviral delivery, electroporation, and ballistic transformation are just a few of the teachings which may be used.

     Transgenic animal: any animal having a cell which includes a DNA

30   sequence which has been inserted by artifice into the cell and becomes part of the genome of the animal which develops from that cell. As used herein, the

transgenic animals are usually mammalian (e.g., rodents such as rats or mice) and the DNA (transgene) is inserted by artifice into the nuclear genome.

Vector: A self-replicating RNA or DNA molecule which can be used to transfer an RNA or DNA segment from one organism to another. Vectors are particularly useful for manipulating genetic constructs and different vectors may have properties particularly appropriate to express protein(s) in a recipient during cloning procedures and may comprise different selectable markers. Bacterial plasmids are commonly used vectors. Modified viruses such as adenoviruses and retroviruses are other examples of vectors.

B) General overview of the invention

The invention generally concerns a protein novel member of the Hypoxia inducible factors (HIFs) HIF-3α. The present inventors have also discovered uses for human HIF-3α proteins, fragments, nucleic acids, and antibodies for modulating HIF-3α cellular levels, for inducing VEGF expression in a mammalian cell, and for inducing angiogenesis in a mammalian tissue. This aspect of the invention also concerns the uses of human HIF-3α proteins, fragments, nucleic acids, and antibodies for modulating HIF-3α cellular levels and for the treatment of coronary and cardiac diseases in mammals, including humans.

The invention also concerns methods and cells, and more particularly genetically modified muscular cells expressing a plurality of angiogenesis-related genes, for inducing angiogenesis and for improving muscular functions. This additional aspect of the invention is based on the use of a nucleotide sequence encoding a transcription factor from the hypoxia inducible factors family (HIF-1α, HIF-2α, and HIF-3α) and provides numerous advantages in the treatment of coronary and cardiac diseases in mammals, including humans.

i) Cloning and molecular characterization of HIF-3α

As it will be described hereinafter in the exemplification section of the invention, the inventors have discovered, cloned and sequenced a human cDNA encoding a new human protein member from the Hypoxia Inducible Factor family, called human HIF-3α (hHIF-3α).

16

The sequence of the HIF-3α cDNA and predicted amino acid sequence is shown in the "Sequence Listing" section. SEQ ID NO: 1 corresponds to the human HIF-3α cDNA and SEQ ID NO: 2 corresponds to the predicted amino acid sequence of the human protein. SEQ ID NO: 3 corresponds to HIF-3α Open reading frame.

The HIF-3α gene encodes a protein of 705 amino acids (A.A.) long. *In silico* analysis indicates that human HIF-3α protein has the following features: it has a molecular weight of about 76 kDa, an isoelectric point of about 5.95; an instability index of about 55.6 (i.e. unstable); an aliphatic index of about 80.6; and a grand average of hydropathicity (GRAVY) of about 0.388. It further comprises many potential phosphorylation sites (45 Ser, 12 Thr, and 3 Tyr) and also many potential phosphorylation sites. Predicted protein domains include the Helix Loop Helix (HLH) heterodimerization domain encoded by amino acids 14-62. This domain is characteristic of HLH transcription factor family. Then, 2 PAS (A.A. 84-143 and 235-289) and 1 PAC (A.A. 295-337) domains are identified, these domains are all common to the PAS family (a sub-family to the HLH factors).

ii) HIF-3α homology with other genes and proteins

The cloning of hHIF-3α was carried out starting with the mouse HIF-3α sequence.

A blast search was made to identify sequence identity between hHIF-3α of the present invention, mHIF-3α and other existing sequences (see Table 1 hereinbelow). It was found that further to mice (GenBank™ accession No. AF060194), HIF-3α had also been sequenced in rat (GenBank™ accession No. NM_022528).

Furthermore, the present inventors also found that their hHIF-3α sequence also shared high level of identity with another, recently cloned hHIF-3α sequence (GenBank™ accession No. AB054067) published by Hara *et al.* (*Biochem. Biophys. Res. Comm.* (2001), Oct 5; 287:808-813). This sequence was published subsequent to the filing date of the application on which the present application claims the benefit. The Hara *et al.* hHIF-3α sequence seems to be another isoform

of HIF-3α, that differs from the hHIF-3α according to the present invention since it is depleted from nucleic acids 1423 to 1545 of SEQ ID NO:1 encoding amino acids 475 to 515 of SEQ ID NO:2. Equivalent mouse and rat sequences also lack amino acids homologues to 475-515 amino acids 475 to 515 of SEQ ID NO:2 and are

5 thus homologues of Hara *et al.* hHIF-3α sequence.. Although not shown, nucleic acids 7 to 345 of SEQ ID NO:1 also shares 100% identity with nucleotides 7 to 345 of a partial cDNA sequence of human HIF-3α published in 1999 (GenBank™ accession No. AF079154), this sequence encoding a 115 amino acid residues (GenBank™ accession No. AAC99397), the last 113 amino acids sharing 100 %

10 identity with amino acids 3 to 115 of SEQ ID NO:2.

TABLE 1: Identified isoform Sequence identity and homology of human

HIF-3α (SEQ ID NOs 1 and 2) to known sequence

Gene	cDNA identity[1]	Amino acid identity	Amino acid similarity
Human HIF-3α[2]	89%	93%	93%
Mouse HIF-3α[3]	74%	77%	82%
Rat HIF-3α[4]	73%	75%	80%

1. *Alignment limited to coding sequence and excluding untranslated regions.*

15 2. *GenBank access AB054067*

3. *GenBank access AF060194*

4. *GenBank access NM_022528*

Therefore, the present invention concerns an isolated or purified nucleic acid

20 molecule (such as cDNA) comprising a sequence selected from the group consisting of :

a) sequences provided in SEQ ID NO: 1 or 3;

b) complements of the sequences provided in SEQ ID NO: 1 or 3;

c) sequences consisting of at least 20 contiguous residues of a sequence

25   provided in SEQ ID NO: 1 or 3;

18

d) sequences that hybridize to a sequence provided in SEQ ID NO: 1 or 3, under moderately or strong stringent conditions;

e) sequences having at least 75% identity to a sequence of SEQ ID NO: 1 or 3; and

5   f) degenerate variants of a sequence provided in SEQ ID NO: 1 or 3.

More preferably, the nucleic acid molecule of the invention comprises a sequence selected from the group consisting of:

a) a nucleotide sequence having at least 75%, 80%, 85%, 90%, 95% or 97% nucleotide sequence identity with SEQ ID NO: 1; and

10   b) a nucleotide sequence having at least 75%, 80%, 85%, 90%, 95% or 97% nucleotide sequence identity with a nucleic acid encoding an amino acid sequence of SEQ ID NO:2.

More preferably, the nucleic acid molecule comprises a sequence substantially the same or having 100% identity with SEQ ID NO: 1 or a sequence

15   substantially the same or having 100% identity with nucleic acids encoding an amino acid sequence of SEQ ID NO: 2. Most preferred nucleic acid molecules are those comprising part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, and/or those comprising part or all of nucleic acids encoding a polypeptide with amino acids 475 to 515 of SEQ ID NO: 2.

20   The present invention also concerns isolated or purified nucleic acid molecules comprising a sequence encoding a human HIF-3α polypeptide or degenerate variants thereof (the human HIF-3α polypeptide or degenerate variant comprising part or all of amino acids 475 to 515 of SEQ ID NO: 2) and purified nucleic acid molecules comprising part or all of nucleic acids 1423 to 1545 of SEQ

25   ID NO: 1.

The present invention also concerns isolated or purified nucleic acid molecule which hybridizes under moderate, preferably high stringency conditions with part or all of any of the HIF-3α nucleic acid molecules of the invention mentioned hereinbefore or with part or all of a complementary sequence thereof.

30   More preferably, the "hybridizing" nucleic acid hybridizes under moderate, preferably high stringency conditions with part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, or with part or all of a complementary sequence thereof. The

19

"hybridizing" nucleic acid could be used as probe or as antisense molecules as it will be described hereinafter.

In a related aspect, the present invention concerns an isolated or purified polypeptide, comprising an amino acid sequence selected from the group
5   consisting of:

   a) sequences encoded by part or all of the HIF-3α nucleic acid molecules of the invention mentioned hereinbefore;

   b) sequences having at least 80% identity to SEQ ID NO: 2;

   c) sequences having at least 85% homology to SEQ ID NO: 2;

10   d) sequence provided in SEQ ID NO: 2;

   e) sequences having at least 80% identity to amino acid sequences encoded by an open reading frame having SEQ ID NO: 3; and

   f) sequences having at least 85% sequence homology to amino acid sequences encoded by an open reading frame having SEQ ID NO: 3.

15   More preferably, the polypeptide comprises an amino acid sequence substantially the same or having 100% identity with SEQ ID NO: 2 or a sequence substantially the same or having 100% identity with amino acid sequences encoded by an open reading frame having SEQ ID NO: 3. Event more preferably, the polypeptides comprise part or all of amino acids 475 to 515 of SEQ ID NO: 2,
20   or degenerate variants thereof, or comprise part or all of amino acids encoded by nucleic acids 1423 to 1545 of SEQ ID NO: 1. Most preferred polypeptides are those having the biological activity of a human HIF-3α polypeptide, such as VEGF expression inducement capabilities for the promotion of angiogenesis.

25   ***iii) Vectors, Cells and Transgenic Animals***

The invention is also directed to a host, such as a genetically modified cell, expressing a functional HIF-3α transcription factor. Preferably, the cell is a skeletal muscular cell or a cardiac cell. Preferably also, the cell comprises a cDNA encoding the transcription factor.

30   The HIF-3α expressing cell may be a transiently-transfected mammalian cell line (such as HEK293 cells, a Hep3B cells, and the like) or any suitable isolated primary cells, including by not limited to mammalian skeletal muscular

20

cells, cardiac cells, bone marrow cells, fibroblasts, smooth muscle cells, endothelial cells, endothelial progenitor cells and embryonic stem cells

A number of vectors suitable for stable transfection of mammalian cells are available to the public (e.g. plasmids, adenoviruses, adeno-associated viruses,
5   retroviruses, Herpes Simplex Viruses, Alphaviruses, Lentiviruses), as are methods for constructing such cell lines. The present invention encompasses any type of vector with a HIF-3α sequence.

The cells of the invention may be particularly useful when transplanted in a compatible recipient for inducing angiogenesis, relieving ischemia, increasing the
10   metabolic activity of a mammalian muscular tissue, and/or increasing muscular function in CHF or in peripheral vascular disease, locally or in surrounding transplanted tissue. Of course, the genetically modified cells of the present invention could also be used for the formation of artificial organs or for tissue constructions. HIF-3α expressing cells may also be used for producing HIF-3α and
15   derivatives thereof (see hereinafter).

The mammalian HIF-3α according to the present invention or a fragment thereof may also be used to generate 1) transgenic animals that express the HIF-3α gene or HIF-3α mutants at various levels in one or multiple cell lineages, 2) knock-out animal in which expression of the endogenous HIF-3α gene is either
20   prevented or regulated in one or multiple cell lineages.

Characterization of HIF-3α genes provides information that is necessary for a HIF-3α knockout animal model to be developed by homologous recombination. Preferably, the model is a mammalian animal, most preferably a mouse. Similarly, an animal model of HIF-3α overproduction may be generated by integrating one or
25   more HIF-3α sequences into the genome, according to standard transgenic techniques.

iv) Synthesis of HIF-3α and functional derivative thereof

Knowledge of human HIF-3α gene sequence open the door to a series of
30   applications. For instance, the characteristics of the cloned HIF-3α gene sequence may be analyzed by introducing the sequence into various cell types or using *in*

21

vitro extracellular systems. The function of HIF-3α may then be examined under different physiological conditions. The HIF-3α cDNA sequence may be manipulated in studies to understand the expression of the gene and gene product. Alternatively, cell lines may be produced which overexpress the gene

5   product allowing purification of HIF-3α for biochemical characterization, large-scale production, antibody production, and patient therapy.

For protein expression, eukaryotic and prokaryotic expression systems may be generated in which the HIF-3α gene sequence is introduced into a plasmid or other vector which is then introduced into living cells. Constructs in which the

10   HIF-3α cDNA sequence containing the entire open reading frame inserted in the correct orientation into an expression plasmid may be used for protein expression. Alternatively, portions of the sequence, including wild-type or mutant HIF-3α sequences, may be inserted. Prokaryotic and eukaryotic expression systems allow various important functional domains of the protein to be recovered

15   as fusion proteins and then used for binding, structural and functional studies and also for the generation of appropriate antibodies.

Eukaryotic expression systems permit appropriate post-translational modifications to expressed proteins. This allows for studies of the HIF-3α gene and gene product including determination of proper expression and post-

20   translational modifications for biological activity, identifying regulatory elements located in the 5' region of the HIF-3α gene and their role in tissue regulation of protein expression. It also permits the production of large amounts of normal and mutant proteins for isolation and purification, to use cells expressing HIF-3α as a functional assay system for antibodies generated against the protein, to test the

25   effectiveness of pharmacological agents or as a component of a signal transduction system, to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring polymorphisms and artificially produced mutated proteins. The HIF-3α DNA sequence may be altered by using procedures such as restriction enzyme digestion, DNA polymerase fill-in,

30   exonuclease deletion, terminal deoxynucleotide transferase extension, ligation of synthetic or cloned DNA sequences and site directed sequence alteration using specific oligonucleotides together with PCR.

22

Accordingly, the invention also concerns a method for producing a human a human HIF-3α polypeptide. The method comprises the steps of: (i) providing a cell transformed with a nucleic acid sequence encoding a human HIF-3α polypeptide positioned for expression in the cell; (ii) culturing the transformed cell under

5   conditions suitable for expressing the nucleic acid; (iii) producing said a human HIF-3α polypeptide; and optionally, (iv) recovering the human HIF-3α polypeptide produced.

Once the recombinant protein is expressed, it is isolated by, for example, affinity chromatography. In one example, an anti-HIF-3α antibody, which may be

10   produced by the methods described herein, can be attached to a column and used to isolate the HIF-3α protein. Lysis and fractionation of HIF-3α-harboring cells prior to affinity chromatography may be performed by standard methods. Once isolated, the recombinant protein can, if desired, be purified further.

Methods and techniques for expressing recombinant proteins and foreign

15   sequences in prokaryotes and eukaryotes are well known in the art and will not be described in more detail. One can refer, if necessary to Joseph Sambrook, David W. Russell, Joe Sambrook Molecular Cloning: A Laboratory Manual 2001 Cold Spring Harbor Laboratory Press. Those skilled in the art of molecular biology will understand that a wide variety of expression systems may be used to produce the

20   recombinant protein. The precise host cell used is not critical to the invention. The HIF-3α protein may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells such as Sf21 cells, or mammalian cells such as COS-1, NIH 3T3, or HeLa cells). These cells are publicly available, for example, from the American Type Culture Collection, Rockville, MD. The

25   method of transduction and the choice of expression vehicle will depend on the host system selected.

Polypeptides of the invention, particularly short HIF-3α fragments, may also be produced by chemical synthesis. These general techniques of polypeptide expression and purification can also be used to produce and isolate useful HIF-3α

30   fragments or analogs, as described herein.

Skilled artisans will recognize that a mammalian HIF-3α, or a fragment thereof (as described herein), may serve as an active ingredient in a therapeutic

23

composition. This composition, depending on the HIF-3α or fragment included, may be used to regulate cell proliferation, survival and angiogenesis and thereby treat any condition that is caused by a disturbance in cell proliferation, accumulation or replacement. Thus, it will be understood that another aspect of

5   the invention described herein, includes the compounds of the invention in a pharmaceutically acceptable carrier.

v) Upregulation of HIF-3α expression for promoting angiogenesis

Knowledge of human HIF-3α gene sequence provides novel promising

10   approaches for patient therapy. As it will be shown in details hereinafter in the exemplification section of the application, upregulation of HIF-3α expression could be used to increase VEGF expression in mammalian cells and thereby promote angiogenesis in ischemic and non-ischemic tissue of mammals, preferably animal models and humans.

15   Therefore, the invention also relates to methods for inducing VEGF expression in a mammalian cell by introducing and expressing in the cell a nucleic acid sequence encoding polypeptide having the biological activity of a human HIF-3α polypeptide. Of course, other nucleic acids, such as those which expression is known to also induce VEGF expression, may be introduced and

20   expressed in the cell together with HIF-3α.

Another related aspect of the invention concerns methods for inducing angiogenesis in a mammalian tissue having a plurality of cells, by introducing and expressing in at least some of these cells a nucleic acid sequence encoding a polypeptide having the biological activity of a human HIF-3α polypeptide.

25   Preferably, these methods are achieved by transfecting *in vitro*, *in vivo* or *ex vivo* cells with a HIF-3α cDNA, the human HIF-3α polypeptide comprises an amino acid sequence substantially the same as SEQ ID NO:2.

As mentioned previously in the "background" section, stimulation of angiogenesis may be beneficial for the treatment of coronary heart diseases.

30   Therefore, the human HIF-3α sequence of the invention could be advantageously used for such purposes. In one embodiment, the cell consists of a cardiac cell located in the heart of a living mammal, and the HIF-3α nucleic acid sequence is

24

introduced in a plurality of these cardiac cell such that expression of the HIF-3α polypeptide induce angiogenesis in cardiac tissue of the mammal. Introduction of the HIF-3α nucleic acid sequence may be done by using a vector as defined previously or by any suitable technique known in the art.

5   In another embodiment, the cell consists of an isolated muscular cell (preferably skeletal muscular cell), and this cell is genetically modified so as to express the HIF-3α polypeptide. Genetically modified HIF-3α expressing cells are then transplanted in tissue (e.g. cardiac tissue) of a compatible mammalian recipient. More preferably, the transplantation is autologous (the cells are isolated 10   from muscular tissue, such as leg, of the recipient), and the cells are transplanted to the recipient (such as an injection in the scar of the heart) in an amount that is sufficient to induce angiogenesis locally or in surrounding transplanted tissue.

In yet another embodiment, the cell consists of a muscular cell located in muscular tissue of a living mammal, and expression of the HIF-3α polypeptide 15   induce angiogenesis in the muscular tissue of the mammal (autologous or heterologous transplantation). This method is particularly useful for treating peripheral artery diseases (e.g. ischemia in the legs due to femoral or upstream artery obstruction in humans).

The nucleotide sequence may be introduced in the cell or tissue using well 20   known methods. Indeed, the sequence(s) may be introduced directly in the cells of a given tissue, injected in the tissue, or introduced via the transplantation of previously genetically modified compatible cells (see hereinafter). Methods for introducing a nucleotide sequence into eukaryote cells such as mammalian muscular cells or for genetically modifying such cells are well known in the art. For 25   instance, this may be achieved with adenoviral vectors, plasmid DNA transfer (naked DNA or complexed with liposomes) or electroporation. If necessary, a person skilled in the art may look at Isner J., (*Nature* (2002), 415:234-239) for a review of myocardial gene therapy methods and to US patent application US20010041679A1 or US patent No. 5,792,453 which provides methods of gene 30   transfer-mediated angiogenesis therapy. Preferably, the level of expression of the transcription factor(s) is such that the angiogenesis-related gene is expressed at a level that is sufficient to induce angiogenesis locally or in surrounding tissue. For

25

better controlling its expression and selectivity, the transcription factor may be inducible.

In preferred embodiments, a plurality of genetically modified skeletal muscular cells are transplanted into the heart of a compatible recipient. Preferably,

5   the transplantation is autologous. More preferably, the cells are transplanted in an amount that is sufficient to induce angiogenesis locally or in surrounding transplanted tissue. Even more preferably, the transplantation improves the recipient's cardiac functions. Transplantation methods, are well known in the art. For detailed examples of muscular cell transplantation, one may refer to US patent

10   Nos. 5,602,301 and 6,099,832.

vi) Downregulation of HIF-3α expression

As mentioned previously, HIF-3α expression induces VEGF expression, which itself promotes angiogenesis. Since it is well known that tumoral cell survival

15   depend on angiogenesis, we propose that, in some tumors, HIF-3α expression is essential for cancer cell proliferation. Accordingly, downmodulation of HIF-3α could be used to prevent and/or treat these tumors.

Therefore, the invention relates to methods for modulating tumoral cell survival or for eliminating a tumoral cell in a human by reducing cellular expression

20   levels of a human HIF-3α polypeptide. In a preferred embodiment, this is achieved by delivering an antisense into the tumoral cells. This can be achieved by intravenous injection, intratumoral injection or other local drug delivery using currently available methods (e.g. Crooke et al., (2000), Oncogene 19, 6651-6659; Stein et al. (2001), J Clin. Invest 108, 641-644; and Tamm et al., (2001), Lancet

25   358, 489-497).

According to a related aspect of the above-mentioned method, the invention relates to antisense nucleic acids and to pharmaceutical compositions comprising such antisenses, the antisense being capable of reducing HIF-3α cellular levels of expression, and more particularly the level of expression of human HIF-3α

30   polypeptide encoded by an open reading frame having SEQ ID NO: 3 and/or comprising amino acids 475 to 515 of SEQ ID NO: 2. Preferably, the antisense nucleic acid is complementary to a nucleic acid sequence encoding a hHIF-3α

protein or encoding any of the polypeptides derived therefrom. More preferably, the antisense hybridizes under high stringency conditions to a genomic sequence or to a mRNA, even more preferably under high stringency conditions with part or all of SEQ ID NO: 1, or with part or all of a complementary sequence thereof. Most

5   preferred antisense molecules are those which hybridize under high stringency conditions with part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, with part or all of nucleic acids 2116 to 2223 of SEQ ID NO: 1, or with part or all of complementary sequences thereof.

A non-limitative example of high stringency conditions includes:

10   a) pre-hybridization and hybridization at 68°C in a solution of 5X SSPE (1X
           SSPE = 0.18 M NaCl, 10 mM NaH_2PO_4); 5X Denhardt solution; 0.05%
           (w/v) sodium dodecyl sulfate (SDS); et 100 µg/ml salmon sperm DNA;
       b) two washings for 10 min at room temperature with 2X SSPE and 0.1%
           SDS;
15   c) one washing at 60°C for 15 min with 1X SSPE and 0.1% SDS; and
       d) one washing at 60°C for 15 min with 0.1X SSPE et 0.1% SDS.

vii) HIF-3α Antibodies

The invention features purified antibodies that specifically binds to a HIF-3α

20   protein. The antibodies of the invention may be prepared by a variety of methods using the HIF-3α proteins or polypeptides described above. For example, the HIF-3α polypeptide, or antigenic fragments thereof, may be administered to an animal in order to induce the production of polyclonal antibodies. Alternatively, antibodies used as described herein may be monoclonal antibodies, which are

25   prepared using hybridoma technology (see, e.g., Hammerling et al., In Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, NY, 1981). The invention features antibodies that specifically bind human HIF-3α polypeptides, or fragments thereof. In particular, the invention features "neutralizing" antibodies. By "neutralizing" antibodies is meant antibodies that interfere with any of the biological activities of

30   the HIF-3α polypeptide, particularly the ability of HIF-3α to induce VEGF expression. The neutralizing antibody may reduce the ability of HIF-3α polypeptides to inhibit VEGF expression by, preferably 50%, more preferably by

27

70%, and most preferably by 90% or more. Any standard assay of VEGF expression, including those described herein, may be used to assess potentially neutralizing antibodies. Once produced, monoclonal and polyclonal antibodies are preferably tested for specific HIF-3α recognition by Western blot,

5   immunoprecipitation analysis or any other suitable method.

In addition to intact monoclonal and polyclonal anti-HIF-3α antibodies, the invention features various genetically engineered antibodies, humanized antibodies, and antibody fragments, including F(ab')2, Fab', Fab, Fv and sFv fragments. Antibodies can be humanized by methods known in the art. Fully

10 human antibodies, such as those expressed in transgenic animals, are also features of the invention.

Antibodies that specifically recognize HIF-3α (or fragments of HIF-3α), such as those described herein, are considered useful to the invention. Such an antibody may be used in any standard immunodetection method for the detection,

15 quantification, and purification of a HIF-3α polypeptide. Preferably, the antibody binds specifically to HIF-3α. The antibody may be a monoclonal or a polyclonal antibody and may be modified for diagnostic or for therapeutic purposes. More preferably the antibody specifically binds the a HIF-3α polypeptide comprising part or all of amino acids 475 to 515 of SEQ ID NO:2. The most preferred antibodies

20 are those that specifically binds to part or all of amino acids 475 to 515 of SEQ ID NO:2.

The antibodies of the invention may, for example, be used in an immunoassay to monitor HIF-3α expression levels, to determine the subcellular location of a HIF-3α or HIF-3α fragment produced by a mammal or to determine

25 the amount of HIF-3α or fragment thereof in a biological sample. Antibodies that inhibit HIF-3α described herein may be especially useful for conditions where decreased HIF-3α function would be advantageous such as inhibition of cancer cell proliferation (see hereinafter). In addition, the antibodies may be coupled to compounds for diagnostic and/or therapeutic uses such as radionucleotides for

30 imaging and therapy and liposomes for the targeting of compounds to a specific tissue location. The antibodies may also be labeled (e.g. immunofluorescence) for easier detection.

28

viii) Administration of HIF-3α Polypeptides, Modulators of HIF-3α Synthesis or Function

Therapies may be designed to circumvent or overcome an inadequate HIF-3α gene expression. This could be accomplished for instance by transfection of HIF-3α cDNA.

To obtain large amounts of pure HIF-3α, cultured cell systems would be preferred. Delivery of the protein to the affected tissues can then be accomplished using appropriate packaging or administrating systems. Alternatively, it is conceivable that small molecule analogs could be used and administered to act as HIF-3α agonists and in this manner produce a desired physiological effect. Methods for finding such molecules are provided herein.

A HIF-3α protein or polypeptide, polypeptide, antibody or modulator (e.g. antisense) may be administered within a pharmaceutically acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be used to provide suitable formulations or compositions to administer HIF-3α protein, polypeptide, or modulator to patients. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful

29

parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and

5   deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

If desired, treatment with a HIF-3α protein, polypeptide, or modulatory compound may be combined with more traditional therapies for the disease such as surgery, steroid therapy, or chemotherapy.

10   According to a preferred embodiment, a HIF-3α antisense would be incorporated in a pharmaceutical composition comprising at least one of the oligonucleotides defined previously, and a pharmaceutically acceptable carrier. The amount of antisense present in the composition of the present invention is a therapeutically effective amount. A therapeutically effective amount of antisense is

15   that amount necessary so that the antisense performs its biological function without causing overly negative effects in the host to which the composition is administered. The exact amount of oligonucleotides to be used and composition to be administered will vary according to factors such as the oligo biological activity, the type of condition being treated, the mode of administration, as well as the other

20   ingredients in the composition. Typically, the composition will be composed of about 1% to about 90% of antisense, and about 20 μg to about 20 mg of antisense will be administered. For preparing and administering antisenses as well as pharmaceutical compositions comprising the same, methods well known in the art may be used. For instance, see Crooke *et al.* (*Oncogene*, 2000, **19**:6651-6659)

25   and Tamm *et al.* (*Lancet* **200**,1358:489-497) for a review of antisense technology in cancer chemotherapy.

ix) Assessment of HIF-3α intracellular or extracellular levels

As noted, the antibodies and probes described above may be used to

30   monitor HIF-3α protein expression and/or to determine the amount of HIF-3α or fragment thereof in a biological sample, and/or to evaluate malignancy of a tumor in a human subject.

30

In addition, *in situ* hybridization may be used to detect the expression of the HIF-3α gene. As it is well known in the art, *in situ* hybridization relies upon the hybridization of a specifically labeled nucleic acid probe to the cellular RNA in individual cells or tissues. Therefore, oligonucleotides or cloned nucleotide (RNA

5   or DNA) fragments corresponding to unique portions of the HIF-3α gene may be used to asses HIF-3α cellular levels or detect specific mRNA species. Such an assessment may also be done *in vitro* using well known methods (Northern analysis, quantitative PCR, etc.).

Determination of the amount of HIF-3α or fragment thereof in a biological

10 sample may be especially useful for diagnosing a cell proliferative disease or an increased likelihood of such a disease, particularly in a human subject, using a HIF-3α nucleic acid probe or HIF-3α antibody. The present inventor also suspect that there exist a correlation between the degree of malignancy of certain types of tumor with the amount HIF-3α or fragment thereof, and that high levels of

15 HIF-3α is indicative that the tumoral cells have a higher angiogenesis activity and malignancy. Highly malignant cancers are cancers which cells display a short doubling time (e.g. hematopoietic cancer, lung cancers, prostate cancer, testis cancer, breast cancer, melanomas, pancreatic cancer intestine cancers, sarcomas, prostate cancer and hematologic cancers).

20 The methods of the invention may be carried out by contacting, *in vitro* or *in vivo*, a biological sample (such as a blood sample or a tissue biopsy) from an individual suspected of harboring cancer cells, with a HIF-3α antibody or a probe according to the invention, in order to evaluate the amount of HIF-3α in the sample or the cells therein. The measured amount would be indicative of the probability of

25 the subject of having proliferating tumoral cells since it is expected that these cells have a higher level of HIF-3α expression.

In a related aspect, the invention features a method for detecting the expression of HIF-3α in tissues comprising, i) providing a tissue or cellular sample; ii) incubating said sample with an anti-HIF-3α polyclonal or monoclonal antibody;

30 and iii) visualizing the distribution of HIF-3α.

Assay kits for determining the amount of HIF-3α in a sample would also be useful and are within the scope of the present invention. Such a kit would preferably comprise HIF-3α antibody(ies) or probe(s) according to the invention and at least one element selected from the group consisting of instructions for
5   using the kit, assay tubes, enzymes, reagents or reaction buffer(s), enzyme(s).

x) Identification of Molecules that Modulate HIF-3α Protein Expression

HIF-3α cDNA may be used to facilitate the identification of molecules that increase or decrease HIF-3α expression. In one approach, candidate molecules
10   are added, in varying concentration, to the culture medium of cells expressing HIF-3α mRNA. HIF-3α expression is then measured (or expression of another gene, such as VEGF which expression is regulated by HIF-3α), for example, by Northern blot analysis using a HIF-3α cDNA, or cDNA or RNA fragment, as a hybridization probe. The level of HIF-3α expression in the presence of the
15   candidate molecule is compared to the level of HIF-3α expression in the absence of the candidate molecule, all other factors (e.g. cell type and culture conditions) being equal.

Compounds that modulate the level of HIF-3α may be purified, or substantially purified, or may be one component of a mixture of compounds such
20   as an extract or supernatant obtained from cells (Ausubel et al., supra). In an assay of a mixture of compounds, HIF-3α expression is tested against progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to modulate
25   HIF-3α expression.

Compounds may also be screened for their ability to modulate HIF-3α-biological activity (e.g. VEGF expression, induction of angiogenesis). In this approach, the biological activity of HIF-3α or of a cell expressing HIF-3α (e.g. lung or kidney cell) in the presence of a candidate compound is compared to the
30   biological activity in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which one or more useful

32

modulator compounds are isolated in a step-wise fashion. The HIF-3α or cell biological activity may be measured by any suitable standard assay.

The effect of candidate molecules on HIF-3α-biological activity may, instead, be measured at the level of translation by using the general approach described above with standard protein detection techniques, such as Western blotting or immunoprecipitation with a HIF-3α-specific antibody (for example, the HIF-3α antibody described herein).

Another method for detecting compounds that modulate the activity of HIF-3α is to screen for compounds that interact physically with a given HIF-3α polypeptide. Depending on the nature of the compounds to be tested, the binding interaction may be measured using methods such as enzyme-linked immunosorbent assays (ELISA), filter binding assays, FRET assays, scintillation proximity assays, microscopic visualization, immunostaining of the cells, in situ hybridization, PCR, etc.

A molecule that promotes an increase in HIF-3α expression or HIF-3α activity is considered particularly useful to the invention; such a molecule may be used, for example, as a therapeutic to increase cellular levels of HIF-3α and thereby exploit the ability of HIF-3α polypeptides to promote and/or induce angiogenesis.

A molecule that decreases HIF-3α activity (e.g., by decreasing HIF-3α gene expression or polypeptide activity) may be used to decrease and/or block angiogenesis and/or cellular proliferation. This would be advantageous in the treatment of cancer.

Molecules that are found, by the methods described above, to effectively modulate HIF-3α gene expression or polypeptide activity, may be tested further in animal models. If they continue to function successfully in an in vivo setting, they may be used as therapeutics to either promotes or inhibit angiogenesis.

33

xi) Induction of Expression of Angiogenesis-related gene(s) by HIF-2α and HIF-3α

According to a related aspect, the present invention relates to methods and cells for inducing angiogenesis and for improving muscular function, and more
5   particularly for treating coronary and cardiac diseases in mammals. The invention also provides genetically modified muscular cells expressing a plurality of angiogenesis-related genes.

This aspect of the invention is based on the use of a nucleotide sequence encoding a transcription factor from the hypoxia inducible factors family (HIF-1α,
10   HIF-2α, and HIF-3α). As it will be shown in the exemplification section, the present inventors have demonstrated that HIF-2α was stimulating, in addition to VEGF, the expression of other molecules implicated in angiogenesis such as IL-8, IL-6, PIGF, LIF receptor, PAI-2 and MMP7 in muscular cells. The inventors showed, in a model of rat CHF, that HIF-2α could be used for therapeutic angiogenesis in
15   mammals. Also, the inventors have genetically modified skeletal muscle cells (SkMC) with HIF-2α gene and showed that these cells demonstrated superior angiogenic properties *in vivo*. Furthermore, treatment of CHF rats with HIF-2α gene transfer resulted in angiogenesis, higher metabolic activity and improved cardiac functions.

20   Since HIF-1α, HIF-2α, and HIF-3α belong to the same family and are closely related, it is expected that similar results could be obtained with any of the HIF members. Therefore, the additional aspects of the present invention given in the present section encompasses not only the use of HIF-2α but HIF-1α and HIF-3α (all isoforms) as well. HIF-1α (GenBank™ accession No. U22431) is
25   described in by Wang *et al.*, (*Proc. Natl. Aca. Sci. USA* (1995) 92: 5510-5514) and in U.S patents No. 5,882,314; 6,020,462 and 6,124,131. HIF-2α (GenBank™ accession No. U81984) is described by Tian *et al.*, (*Genes & Dev.* (1996) 11: 72-82), and in U.S patent No. 5,692,963. One isoform of HIF-3α is described herein and another isoform (GenBank™ accession No. AB054067) has been described
30   by Hara *et al.* (*Biochem. Biophys. Res. Comm.* (2001), Oct 5; 287:808-813).

34

Therefore, this aspect of the invention is directed to a method for inducing in a muscular mammalian cell the expression of at least one, preferably a plurality of angiogenesis-related gene(s), the method comprising the step of introducing and expressing in the cell a nucleic acid sequence encoding a functional HIF-2α

5   transcription factor or a functional HIF-3α transcription factor.

According to another related aspect, the invention is directed to a method for increasing the metabolic activity of a muscular cell (such as glucose consumption), comprising the step of introducing and expressing in the cell a nucleic acid sequence encoding a functional transcription factor of the Hypoxia

10 Inducible Factor (HIF) family. Preferably, the transcription factor is HIF-2α or HIF-3α.

In a further related aspect, the invention is directed to a method for improving cardiac tissue functions of a mammal, comprising the step of providing to the cardiac tissue of the mammal a plurality of genetically modified cells

15 expressing a nucleic acid sequence encoding a functional HIF-2α transcription factor or a functional HIF-3α transcription factor.

According to another related aspect, the invention is directed to a method for inducing angiogenesis in a mammalian muscular tissue, comprising the step of providing the muscular tissue with a plurality of genetically modified muscular cells

20 expressing a nucleic acid sequence encoding a functional HIF-2α transcription factor or a functional HIF-3α transcription factor.

According to the invention, a nucleotide sequence encoding a transcription factor of hypoxia inducible factor family is introduced and expressed into a muscular cell, preferably a skeletal muscular cell or a cardiac cell by using any

25 suitable method including but not limited to adenoviral infection, and plasmid, cosmid or artificial chromosome transfection or electroporation. More preferably, the nucleic acid sequence encoding the transcription factor(s) is a cDNA.

In a further aspect, the invention is directed to a genetically modified muscular cell (e.g. skeletal muscle cell, cardiac cell) expressing a functional

30 HIF-2α transcription factor or a functional HIF-3α transcription factor. Preferably, the cell is a skeletal muscular cell or a cardiac cell. Preferably also, the cell comprises a cDNA encoding the transcription factor. As mentioned previously,

35

such cells may be particularly useful when transplanted in a compatible recipient for inducing angiogenesis, relieving ischemia, increasing the metabolic activity of a mammalian muscular tissue, and/or increasing muscular function in CHF or in peripheral vascular disease, locally or in surrounding transplanted tissue. Of

5   course, the genetically modified cells of the present invention could also be used for the formation of artificial organs or for tissue constructions.

Although transplantation of cells (autologous transplantation or from a compatible donor) is preferred for inducing angiogenesis locally, in surrounding tissue and/or for improving the metabolic activity of a muscular cell and/or for

10 relieving ischemia in coronary heart disease or in peripheral vascular disease and/or for improving the mammal's cardiac functions, the nucleic acid sequence encoding the functional transcription factor may be introduced directly in the tissue of the mammal using any suitable method known in the art (see hereinbefore for some examples). The angiogenesis-related gene should be expressed at a level

15 that is sufficient to induce angiogenesis locally or in surrounding tissue.

As it will now be demonstrated by way of an example hereinafter, the present invention is useful for inducing angiogenesis, relieving ischemia and increasing cell metabolic activity and tissue function in CHD and in PVD.

20   **EXAMPLES**

The following examples are illustrative of the wide range of applicability of the present invention and is not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any method and material similar or equivalent to those

25 described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described.

36

EXAMPLE 1: Use of HIF-1α, HIF-2α and HIF-3α for inducing angiogenesis and improving muscular functions

Materials and methods

Plasmids construction

5   HIF-1α-VP16/pcDNA3 was obtained from S.L. McKnight (Tian *et al.*, *Genes & Dev.* (1996) 11:72-82). To generate HIF-1α-VP16/pcDNA3, a VP16 fragment (*Nhe*I and blunt ended *EcoR*I from pVP16 (ClonTech)) was inserted in HIF-1α/pcDNA3 cut with *Afl*II and *Xba*I blunt ended in presence of a *Nhe*I-*Afl*II linker composed of the oligos TTA AGA TAT CGA TGA CAC GTG (SEQ ID NO:4) and

10   TCA GCA CGT GTC ATC GAT ATC (SEQ ID NO:5), replacing the 3' part of HIF-1α sequence by VP16, putting DNA binding domain and heterodimerization domains (HLH and PAS) of HIF-1α (encoded by the 5' of the gene) in frame with VP16 transcription activation domain.

HIF-2α-VP16/pcDNA3 was obtained from S.L. McKnight (Tian *et al.*, *Genes*

15   *& Dev.* (1996) 11:72-82). To generate HIF-2α-VP16/pcDNA3, a HIF-2α 5' region was amplified by PCR using HIF-2α/pcDNA3 as template with the oligonucleotides T7 and GCT AGC TAG GAA GTT ACT CCT CTC (SEQ ID NO:6), creating a *Nhe*I at the end of HIF-2α DNA binding domain and heterodimerization domains (HLH-PAS). This fragment was cut with *Nhe*I and *Kpn*I and was inserted in place of HIF-

20   1α sequence in HIF-1-VP16/pcDNA3 cut *Nhe*I and *Kpn*I. Sequencing confirmed integrity of the amplified sequence.

HIF-3α was cloned by RT-PCR by homology deduction from mouse HIF-3α sequence. Using Marathon™ RT-PCR kit (ClonTech), 2 fragments of HIF-3α sequence were amplified from adult human heart RNA (ClonTech) with the oligos

25   CCA TGG ACA GGT CGA CCA CGG AGC TGC GCA GG (SEQ ID NO:7) (containing an ATG initiator in a *Nco*I site) and CGC AGG CAG GTG GCT TGT AGG CCC T (SEQ ID NO:8) for the 5' end and with the oligos CAG CTG GAG CTC ATT GGA CAC AGC ATC (SEQ ID NO:9) and CCC CAT CCT GTG CGT TGG CTG CCG (SEQ ID NO:10) for the 3' end. Both fragments were sequenced

30   and put together with the unique *Nde*I site, and the reconstructed cDNA was inserted in the *Nco*I initiator of HIF-1α/pcDNA3, using HIF-1α Kozak sequence to

initiate translation. To generate HIF-3α-VP16/pcDNA3, a PCR fragment containing HIF-3α DNA binding domain and heterodimerization domains (HLH and PAS) was amplified using HIF-3α/pcDNA3 as template with the oligos T7 and GGA GTC AGC TTA AGC TGA ATG GGT CTG C (SEQ ID NO:11). The amplification product
5   was cut with BamHI and AflII (coming from the 3' oligo) and inserted in place of HIF-1α sequence in HIF-1α-VP16/pcDNA3 cut with BamHI and AflII. Sequencing confirmed integrity of the amplified sequence.

Transfection

10   Early passage 293 cells (ATCC # CRL-157) were plated at 1.7×10^6 cells per plate (100 mm) and grown overnight. 10 µg of sterile plasmid DNA was transfected with lipofectamin, according to the instructions from the manufacturer. After a 5 hours transfection, cells were incubated either in normoxia (5% CO_2 in normal air at 37°C) or in hypoxia (5% CO_2, 2% O_2 at 37°C) for 24 hours. ~70%
15   confluence Hep3B cells (ATCC #HB-8064) in 100 mm dishes were transfected with 4 µg of sterile plasmid DNA with 6 µl of Fugene 6™ (Roche), according to the instructions from the manufacturer.

Adenovirus production

20   HIF constructs were used to produce adenoviral vectors with the Ad.Easy™ technology using manufacturer methodology (Q-Biogene).

Infection

Early passage human SkMC (Cambrex #CC-2561) were plated in 100 mm
25   dishes and grown until they reached ~70% confluence. Cells were rinsed with PBS and covered with 4 ml DMEM with 10% fetal calf serum (FCS) and adenoviruses at a MOI of 500. This MOI allow infection of only ~10% of the cells. Cells were incubated at 37°C with constant but gentle agitation for 6 hours. 6 ml of DMEM with 10% FCS was added and cells were incubated overnight at 37°C. Cells were
30   then incubated either in normoxia (5% CO_2 in normal air at 37°C) or in hypoxia (5% CO_2, 2% O_2 at 37°C) for 24 hours.

38

RNAses Protection Assay (RPA)

Total RNA was isolated from transduced cells by Guanidine thiocyanate-phenol extractions as described (Staffa, A., et al., *J. Biol. Chem.* (1997) **272**: 33394-401) (Figure 3A or 3C) or with Rneasy Mini Kit™ (Qiagen) as described by

5   the manufacturer. 15 µg total RNA per sample were analyzed by RPA using RPA III kit (Ambion). Probe were prepared from hAngio1 template set (Pharmingens) for Figure 3A or from home made VEGF and actin template for the others. VEGF template was prepared by amplification on the phVEGF165.SR (from J.F. Isner) (Tsurumi, Y., et al., *Circulation.* (1996) **94**: 3281-3290) with the oligos CCG GAA

10   TTC TCT ACC TCC ACC ATG CC (SEQ ID NO:12) and CCG GAA TTC CTC AGT GGG CAC ACA CTC C (SEQ ID NO:13), digestion of the amplification product with *Eco*RI (in both oligos) and insertion in pBluescript cut with *Eco*RI. Sequence integrity was confirmed by sequencing. The resulting plasmid was digested with *Hind*III and transcribed with T7 RNA polymerase to generate an antisense RNA

15   probe. Actin probe template was obtained from W.E. Bradley (Houle, B., *et al.*, *Proc. Natl. Aca. Sci. USA* (1993) **90**: 985-989).

Quantification

Autoradiograms were scanned on an Alpha Imager 2000™ (Alpha Innotech

20   Corporation). Intensity of each band was measured and relative expression was calculated as VEGF intensity/Flt4 intensity for Figure 3A or as VEGF intensity/actin intensity for the others. Relative VEGF values were then normalized so that a value of 100 was attributed to the control samples in normoxia. Protein VEGF produced were proportional to the amount of mRNA quantified. Results are

25   expressed as mean ± SED.

Gene chip hybridization

Total RNA was isolated from human SkMC (Clonetics) infected with either Ad.Null™ (Q-Biogene) or Ad.HIF-2α as described (Staffa, *et al.*, *J. Biol. Chem.*

30   (1997) **272**: 33394-401). Probes were prepared and hybridized to Atlas Human 1.2 Array™ (Clontech) with ExpressHyb™ solution (Clontech) according to the

instructions from the manufacturer. The arrays were exposed to phosphorimager screen and analyzed with the Atlas 2.01™ software (Clontech).

Angiogenesis in vivo

5   Early passage human SkMC (uninfected or infected with the indicated adenovirus vectors) were incorporated into matrigel (50000 cells/0.5 ml) and injected subcutaneously into skid mice (10 per groups). Matrigel alone or bFGF (400 ng/ml) were used as controls. 7 days post-implantation, matrigel plugs were excised and processed for histological evaluation. Each measurement

10   corresponds to the degree of cellular response in a microscopic field expressed as Integrated Density (sum of the gray values in the selection, with the background subtracted, 6 measurements on each plug).

Metabolic activity in infarcted rats created by a permanent left anterior descending coronary artery ligation (Myoinfarct™ rats, Charles River Laboratories)

15   was measured 5 days post ligature by injection of ^{18}FDG (~750 µCi / 100 g) and dynamic acquisition using a small animal PET-Scan™ (Sherbrooke University). Ten days after the ligature, a mini-thoracotomy was performed to inject a solution of Ad.HIF2α/VP16 or saline (N=2) in the infarcted area of the left ventricle. Another ^{18}FDG PET-Scan™ was performed 14, 28, 42 and 56 days after the injection.

20   Metabolic activity was calculated as the amount of ^{18}FDG found in an area of the lateral wall of the left ventricle, where the infarct is localized, normalized with the amount of FGD found in an unaffected area in the septal wall that is irrigated by another coronary artery. After the last PET-scan™, animals were sacrificed and blood vessels were quantified in the entire infarcted area or in the fields of the

25   margins between healthy and infarcted tissues.

Results

In vitro VEGF induction by HIF-1α, HIF-2α and HIF-3α

Figures 2A, 2B, 3A, 3B and 3C illustrate the expression of angiogenesis

30   related genes in human cells transfected with HIF constructs.

The natural (wild type) form of HIF factors or activated version were introduced in pcDNA3 (Invitrogen Corp.), an expression vector. Activated

40

constructs consist in the deletion of hypoxia regulated instability domains located in the N' part of the proteins and the introduction of a strong activator of transcription, the VP16 domain from Herpes virus (Vincent et al., Circulation (2000) **102**: 2255-2261). The resulting hybrids are no longer regulated by hypoxia and always display a transactivation activity.

These plasmids were transiently transfected in human embryonic kidney 293 cells (HEK293) or in human liver cells Hep3B. Cells were incubated 24 hours either in normoxia (normal conditions) or in hypoxia (2% oxygen) and total RNA was isolated. Similarly, human skeletal muscle cells (hSkMC) were transduced with adenovirus vectors expressing some of the HIF constructs. Endogenous VEGF gene activation following HIF constructs transfection was analyzed by RNAse Protection Assay (RPA, Ambion).

No VEGF induction was produced by HIF-1α transfection in normoxia (similar to control) (Figures 2A, 2B, 3A and 3B). This result was expected since HIF-1α is quickly degraded in normoxia. The HIF-1α/VP16 hybrid stimulated VEGF both in normoxia and hypoxia, since the protein is no longer regulated by oxygen tension. Interestingly, HIF-3α was very efficient to stimulate VEGF in HEK293 and Hep3B cells (Figure 2A and 2B). The levels were superior to those obtained with HIF-1α both in normoxia and in hypoxia, although HIF-1α/VP16 had an even higher transactivation activity. The activation of HIF-3α with the VP16 domain did not significantly modify the levels of VEGF induction.

VEGF stimulation by HIF-2α was also very important. Figure 3A, 3B and 3C show that the wild type version of the protein stimulated VEGF in ranges comparable to HIF-1α/VP16, both in normoxia and in hypoxia, in the 3 cells types studied (HEK293 in Figure 3A, Hep3B in Figure 3B and hSKMC in Figure 3C). The VP16 fusion did not provide an important advantage in the levels of VEGF produced.

Activation of angiogenic genes by HIF-2α in vitro

VEGF is an important angiogenic gene. However, angiogenesis is a complex process involving several steps that are regulated by several factors. To evaluate HIF factors potential as an angiogenic modulator, gene expression was

compared in human SkMC infected either with Ad.HIF2α or Ad.Null™ using gene chip technology. cDNA probes derived from either cell population was hybridized on a Atlas Human 1.2 Array™ (Clontech) assessing expression of almost 1200 genes. Of the genes differentially regulated, VEGF was the one showing the most

5   significant increase. VEGF stimulates both proliferation, migration and differentiation of endothelial cells. Interestingly, other angiogenic genes were activated (Table 2). As VEGF, Interleukin-8 (IL-8) and the activation of Leukemia Inhibitory Factor Receptor (LIF-R) are known to stimulate the proliferation of endothelial cells. However, IL-8 also acts by activating the release of

10   metalloproteinases responsible for the basal membrane disruption, the first step of angiogenesis (Figure4). LIF is also known to enhance survival of SkMC, which would be useful in cell therapy. The role of Interleukin-6 (IL-6) is more indirect: it has no effect on endothelial cell proliferation, but stimulates their migration and differentiation. It is also an enhancer of VEGF production. Placental growth factor

15   (PIGF) was shown to act in cooperation with VEGF to stimulate angiogenesis. Matrix metalloproteinase 7 (MMP7) is a potent proteinase able to initiate the angiogenesis process while placental plasminogen activator inhibitor 2 (PAI-2) is able to stabilize nascent vessels (Figure 4). There are many other potential angiogenic factors that couldn't be detected in this assay because of limitations of

20   the gene chip, but that might be induced by HIF factors.

42

Table 2: G nes activated by HIF-2α in SkMC.

Gen	Fold induction	Category
VEGF	9.10	Growth factor
IL-8	2.26	Growth factor
IL-6	2.21	Growth factor
PlGF	Up*	Growth factor
LIF-R	Up*	Growth factor
PAI-2	1.93	Proteinase inhibitor
MMP7	Up*	Metalloproteinase

*Inductions labeled "up" are representing the activation from a previously undetected gene.

5   *HIF-2α stimulates angiogenesis in vivo*

Angiogenic potential of HIF constructs were evaluated *in vivo* by subcutaneous implantation of SkMC transfected with HIF constructs in mice. Seven days following implantation, modified cells pellets showed significantly higher blood vessel density than unmodified SkMC (Figure 5B). This result
10   confirms that the VP16 activation is not necessary in the case of HIF-2α. Modified SkMC differentiated *in vivo* into myotubes supported by extracellular matrix and surrounded by new blood vessels (Figure 5A). The angiogenesis was important around the myotubes and appeared well organized.

HIF-2α was also directly delivered in a model of MI in rats. Metabolic activity
15   was assayed 5 days before direct myocardial injection of the vector Ad.HIF-2α/VP16 and at several time points in the following 2 months. As shown in Figures 6A and 6B, an improved metabolic activity was measured in the infarcted area of treated rats over control ones as quantified by position emission tomography-scan (PET-Scan™). When blood vessel density was quantified by
20   histology, an increase number of blood vessels was noted for the adenovirus treated rats in the infarcted area, as well as in the peri-infarcted zone (Figure 6B).

43

3) Discussion

In vitro transfection experiments showed that wild type HIF-3α and HIF-2α were superior to HIF-1α to induce VEGF. Used in gene therapy, HIF-3α would thus be very useful to modulate angiogenesis.

5   As shown in Figures 2A, 2B, 3A, 3B and 3C, transfection of HIF-VP16 fusion encoding constructs resulted in a significant stimulation of VEGF. However, in gene therapy in humans, these constructs pose the problem of immunogenicity. The presence of the VP16 sequence, of viral origin, is recognized by the immune system and trigger an immune response against the transferred gene. Construct

10   using wild type human genes, such as HIF-3α, has the advantage of being less immunogenic, thus providing a longer period of expression. In comparison, HIF-1α had a poor angiogenic potential in normoxia and requires the VP16 modification to be fully active (Figures 2, 3 and Vincent et al., Circulation (2000) 102: 2255-2261). Figures 2A, 2B, 3A, 3B and 3C also strongly suggest that HIF-2α and HIF-3α

15   could be used efficiently in gene transfer protocols to modify gene transcription of the targeted cells. In particular, VEGF expression is increased, which result in angiogenesis. It is thus proposed to use HIF-2α or HIF-3α sequence in ischemic diseases to increase blood vessels and blood perfusion.

The analysis of genes activated by HIF-2α revealed the induction of several

20   angiogenic genes (Table I). These genes play a role in various aspects of angiogenesis (Figure 4) and the resulting angiogenesis is thus expected to be strong and well organized. This is a major advantage compared to the use of solely VEGF to induce angiogenesis since all the key steps of angiogenesis will be stimulated. It is expected that HIF-3α will also regulate several angiogenesis

25   related genes, and possibly the same ones as HIF-2α.

In vivo experiments proved that HIF-2α gene transfer resulted in a significant angiogenesis, both in sub-cutaneous implants (Figure 5B) and in a MI heart model (Figure 6B). These results confirmed that the VP16 fusion was not necessary to result in the formation on blood vessels. This characteristic confers

30   an important advantage to HIF-2α sequence as an angiogenic agent. The use of a native sequence in gene transfer therapy will raise a much lower immunogenic response than a construct with VP16, of viral origin. Since HIF-3α was also

44

superior to HIF-1α and showed *in vitro* induction of VEGF similar to HIF-2α, it is expected that it will also be efficient *in vivo*.

Angiogenesis resulted in an increase in metabolic activity (increased glucose consumption) in the infarcted area as shown in Figure 6A, indicating an
5   improvement in the tissue function. This, higher blood supply translated in higher muscle activity which can improve heart pumping function.

In summary, it is clear that HIF factors delivered either directly or via an implanted cell, offers a great potential for myocardial regeneration and improvement of cardiac function.

10 The results reported herein constitute a proof of principle to the effect that HIF-2α and HIF-3α can be used in gene therapy.

While several embodiments of the invention have been described, it will be understood that the present invention is capable of further modifications, and this
15 application is intended to cover any variations, uses, or adaptations of the invention, following in general the principles of the invention and including such departures from the present disclosure as to come within knowledge or customary practice in the art to which the invention pertains, and as may be applied to the essential features hereinbefore set forth and falling within the scope of the
20 invention or the limits of the appended claims.

45

CLAIMS:

1.   An isolated or purified nucleic acid molecule comprising a sequence
selected from the group consisting of :

5   a) sequences provided in SEQ ID NO: 1 or 3;

b) complements of the sequences provided in SEQ ID NO: 1 or 3;

c) sequences consisting of at least 20 contiguous residues of a sequence
provided in SEQ ID NO: 1 or 3;

d) sequences that hybridize to a sequence provided in SEQ ID NO: 1 or 3, under

10   moderately stringent conditions;

e) sequences having at least 75% identity to a sequence of SEQ ID NO: 1 or 3;
and

f) degenerate variants of a sequence provided in SEQ ID NO: 1 or 3.

15   2.   The nucleic acid of claim 1, wherein it comprises a sequence selected from
the group consisting of:

a) a nucleotide sequence having at least 75% nucleotide sequence identity with
SEQ ID NO: 1; and

b) a nucleotide sequence having at least 75% nucleotide sequence identity with a

20   nucleic acid encoding an amino acid sequence of SEQ ID NO:2.

3.   The nucleic acid of claim 1 or 2, wherein it comprises a sequence selected
from the group consisting of:

a) a sequence substantially the same as SEQ ID NO: 1; and

25   b) a sequence substantially the same as a nucleic acid encoding an amino acid
sequence of SEQ ID NO: 2.

4.   The nucleic acid of any one of claims 1 to 3, wherein it comprises a
sequence selected from the group consisting of:

30   a) a sequence having 100% identity with SEQ ID NO: 1;

b) a sequence having 100% identity with a nucleic acid encoding an amino acid
sequence of SEQ ID NO: 2.

46

5.   The nucleic acid molecule of any one of claims 1 to 4, wherein it comprises:

a) part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1;

b) part or all of nucleic acids encoding a polypeptide with amino acids 475 to 515

5   of SEQ ID NO: 2.

6.   The nucleic acid molecule of any one of claims 1 to 5, wherein it encodes a

polypeptide having the biological activity of a human HIF-3α polypeptide.

10   7.   An isolated or purified nucleic acid molecule comprising a sequence

encoding a human HIF-3α polypeptide or degenerate variants thereof, wherein

said human HIF-3α polypeptide or degenerate variant comprises part or all of

amino acids 475 to 515 of SEQ ID NO: 2.

15   8.   The nucleic acid molecule of claim 7, wherein it comprises part or all of

nucleic acids 1423 to 1545 of SEQ ID NO: 1.

9.   The nucleic acid molecule of claim 7 or 8, wherein it comprises SEQ ID

NO: 1.

20

10.   The nucleic acid of any one of claims 1 to 9, wherein said nucleic acid

molecule consists of a cDNA.

11.   An isolated or purified nucleic acid molecule which hybridizes under high

25   stringency conditions with part or all of any of the nucleic acid molecules of claims

7 to 10, or with part or all of a complementary sequence thereof.

12.   The nucleic acid molecule of claim 11, wherein it hybridizes under moderate

or high stringency conditions with part or all of nucleic acids 1423 to 1545 of SEQ

30   ID NO: 1, or with part or all of a complementary sequence thereof.

47

13.   An isolated or purified protein comprising an amino acid sequence selected from the group consisting of:

a) sequences encoded by a nucleic acid of any one of claims 1 to 12;

b) sequences having at least 80% identity to SEQ ID NO: 2;

5   c) sequences having at least 85% homology to SEQ ID NO: 2;

d) sequence provided in SEQ ID NO: 2;

e) sequences having at least 80% identity to amino acid sequences encoded by an open reading frame having SEQ ID NO: 3; and

f) sequences having at least 85% sequence homology to amino acid sequences

10   encoded by an open reading frame having SEQ ID NO: 3.

14.   The protein of claim 13, wherein it comprises an amino acid sequence selected from the group consisting of:

a) sequences substantially the same as SEQ ID NO: 2; and

15   b) sequences substantially the same as amino acid sequences encoded by an open reading frame having SEQ ID NO: 3.

15.   The protein of claim 13 or 14, wherein it comprises an amino acid sequence 100% identical to SEQ ID NO: 2.

20

16.   The protein of any one of claims 13 to 15, wherein said protein has the biological activity of a human HIF-3α polypeptide.

17.   The protein of claim 16, wherein said biological activity comprises inducing

25   VEGF expression.

18.   The protein of claim 17, wherein inducement of VEGF expression promotes angiogenesis.

30   19.   An isolated or purified human protein comprising part or all of amino acids 475 to 515 of SEQ ID NO: 2, or degenerate variants thereof.

48

20.   The human protein of claim 19, wherein said protein has the biological activity of a human HIF-3α polypeptide.

21.   The protein of claim 20, wherein said biological activity comprises inducing·
5   VEGF expression.

22.   An antisense nucleic acid that reduces a human HIF-3α polypeptide levels of expression.

10   23.   The antisense of claim 22, wherein said human HIF-3α polypeptide comprise amino acids 475 to 515 of SEQ ID NO: 2.

24.   The antisense of claim 22 or 23, wherein said human HIF-3α polypeptide is encoded by an open reading frame having SEQ ID NO: 3.
15

25.   The antisense any one of claims 22 to 24, wherein said antisense hybridizes under high stringency condition with part or all of SEQ ID NO: 1, or with part or all of a complementary sequence thereof.

20   26.   The antisense nucleic acid any one of claims 22 to 25, wherein said antisense hybridizes under high stringency conditions with part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, or with part or all of a complementary sequence thereof.

25   27.   The antisense nucleic acid of any one of claims 22 to 25, wherein said antisense hybridizes under high stringency conditions with part or all of nucleic acids 2116 to 2223 of SEQ ID NO: 1, or with part or all of a complementary sequence thereof.

30   28.   The antisense of any one of claims 22 to 27, wherein said antisense hybridizes under high stringency conditions to a genomic sequence or to a mRNA. ·

49

29.   An isolated or purified antibody that specifically binds to a HIF-3α polypeptide substantially the same as SEQ ID NO:2.

30.   The antibody of claim 29, wherein said antibody specifically binds to a
5   HIF-3α polypeptide comprising part or all of amino acids 475 to 515 of SEQ ID NO:2.

31.   The antibody of claim 29 or 30, wherein said antibody specifically binds to part or all of amino acids 475 to 515 of SEQ ID NO:2.
10

32.   The antibody any one of claims 29 to 31, wherein said antibody consists of a monoclonal or of a polyclonal antibody.

33.   A pharmaceutical composition comprising:
15   1) at least one element selected from the group consisting of:
            i)   a nucleic acid molecule encoding a human HIF-3α polypeptide;
            ii) a human HIF-3α polypeptide;
            iii) an antisense nucleic acid that reduces a human HIF-3α polypeptide levels of expression; and
20   iv) an isolated or purified antibody that specifically binds to a HIF-3α polypeptide;
        and
        2) a pharmaceutically acceptable carrier.

25   34.   The composition of claim 33, wherein said human HIF-3α polypeptide has an amino acid sequence substantially the same as SEQ ID NO:2.

35.   The composition of claim 33 or 34, wherein said human HIF-3α polypeptide comprises amino acids 475 to 515 of SEQ ID NO:-2.
30

36.   A cloning or expression vector comprising the nucleic acid of any one of claims 1 to 12.

50

37.   The vector of claim 36, wherein said vector is capable of directing expression of the peptide encoded by said nucleic acid in a vector-containing cell.

5   38.   The vector of claim 36 or 37, wherein said vector is selected from the group consisting of plasmids, adenoviruses, adeno-associated viruses, retroviruses, Herpes Simplex Viruses, Alphaviruses, and Lentiviruses.

39.   A transformed or transfected cell that contains the nucleic acid of any one of
10   claims 1 to 12.

40.   The cell of claim 39, wherein said cell consists of a cell selected from the group consisting of HEK293 cells, Hep3B cells, mammalian skeletal muscular cells, cardiac cells, bone marrow cells, fibroblasts, smooth muscle cells,
15   endothelial cells, endothelial progenitor cells and embryonic stem cells.

41.   A transgenic animal generated from the cell of claim 39 or 40, wherein said nucleic acid is expressed in said transgenic animal.

20   42.   A nucleotide probe comprising a sequence of at least 15 sequential nucleotides of SEQ ID NO: 1 or of a sequence complementary to SEQ ID NO: 1.

43.   The nucleotide probe of claim 42, comprising part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1, part or all of nucleic acids 2116 to 2223 of SEQ ID
25   NO: 1, part or all of nucleic acids encoding amino acids 475 to 515 of SEQ ID NO: 2, or part or all of a complementary sequence thereof.

44.   A method for inducing VEGF expression in a mammalian cell, the method comprising step of introducing and expressing in said cell a nucleic acid sequence
30   encoding polypeptide having the biological activity of a human HIF-3α polypeptide.

45.   The method of claim 44, wherein said human HIF-3α polypeptide comprises an amino acid sequence substantially the same as SEQ ID NO:2.

46.   The method of claim 44 or 45, wherein said human HIF-3α polypeptide

5   comprises part or all of amino acids 475 to 515 of SEQ ID NO: 2.

47.   The method of claim any one of claims 44 to 46, wherein a nucleic acid sequence comprises part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1.

10   48.   The method any one of claims 44 to 47, wherein said cell consists of a cardiac cell located in the heart of a living mammal, and wherein expression of said polypeptide induce angiogenesis in cardiac tissue of said mammal.

49.   The method any one of claims 44 to 47, wherein said cell consists of a

15   muscular cell located in muscular tissue of a living mammal, and wherein expression of said polypeptide induce angiogenesis in the muscular tissue of said mammal.

50.   The method any one of claims 44 to 47, further comprising the step of

20   transplanting said cell in tissue of a compatible mammalian recipient.

51.   The method of claim 50, wherein said cells are transplanted in an amount that is sufficient to induce angiogenesis locally or in surrounding transplanted tissue.

25

52.   The method of claim 51, wherein said transplanted tissue consists of an ischemic or a non-ischemic tissue.

53.   The method of any one of claims 44 to 47, wherein said mammalian cell is a

30   skeletal muscular cell thereby providing a HIF-3α expressing-skeletal muscular cell, and wherein said method further comprises the step of transplanting a

52

plurality of said HIF-3α expressing-skeletal muscular cells in a cardiac tissue of a compatible mammalian recipient.

54.   The method of any one of claims 50 to 53, wherein said the transplantation
5   step consists of an autologous transplantation and wherein said mammalian recipient is a human.

55.   A method for inducing angiogenesis in a mammalian tissue having a plurality of cells, the method comprising the step of introducing and expressing in
10   at least some of said cells a nucleic acid sequence encoding a polypeptide having the biological activity of a human HIF-3α polypeptide.

56.   The method of claim 55, wherein said human HIF-3α polypeptide comprises an amino acid sequence substantially the same as SEQ ID NO:2.
15

57.   The method of claim 55 or 56, wherein said human HIF-3α polypeptide comprises part or all of amino acids 475 to 515 of SEQ ID NO: 2.

58.   The method of any one of claims 55 to 57, wherein said nucleic acid
20   sequence comprises part or all of nucleic acids 1423 to 1545 of SEQ ID NO: 1.

59.   A method for modulating tumoral cell survival or for eliminating a tumoral cell in a mammal, comprising the step of reducing cellular expression levels of a HIF-3α polypeptide.
25

60.   The method of claim 59, wherein said mammal consists of a human, and wherein the method comprises delivering a human HIF-3α antisense into said tumoral cell.

30   61.   A method for determining the amount of a human HIF-3α polypeptide or a human HIF-3α nucleic acid in a biological sample, comprising the step of

53

contacting said sample with the antibody of any one of claims 29 to 32 or with a probe of any one of claims 42 or 43.

62.   A method of evaluating malignancy of a tumor in a human subject,
5   comprising the step of determining the amount of a HIF-3α polypeptide or of a HIF-3α nucleic acid in a tumoral cell from said subject, wherein said amount is indicative of a degree of malignancy for said tumor.

63.   A kit for determining the amount of a human HIF-3α polypeptide or a human
10   HIF-3α nucleic acid in a biological sample, said kit comprising the antibody of any one of claims 29 to 32 or with a probe of any one of claims 42 or 43, and at least one element selected from the group consisting of instructions for using said kit, reaction buffer(s), and enzyme(s).

15   64.   A method for producing a human HIF-3α polypeptide comprising:
       -    providing a cell transformed with a nucleic acid sequence encoding a human HIF-3α polypeptide positioned for expression in said cell;
       -    culturing said transformed cell under conditions suitable for expressing said nucleic acid; and
20   -   producing said human HIF-3α polypeptide.

hHIF-3a   (2223 bps)
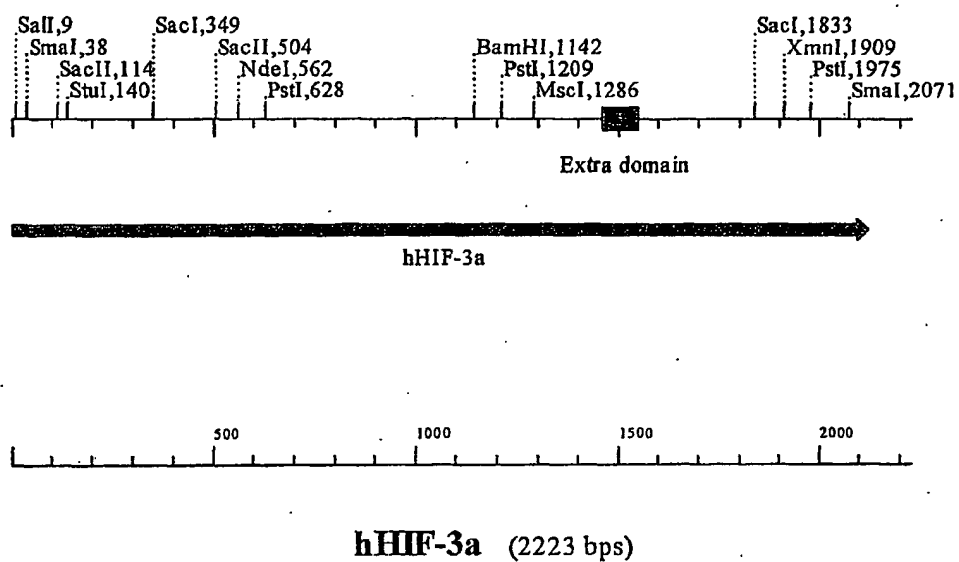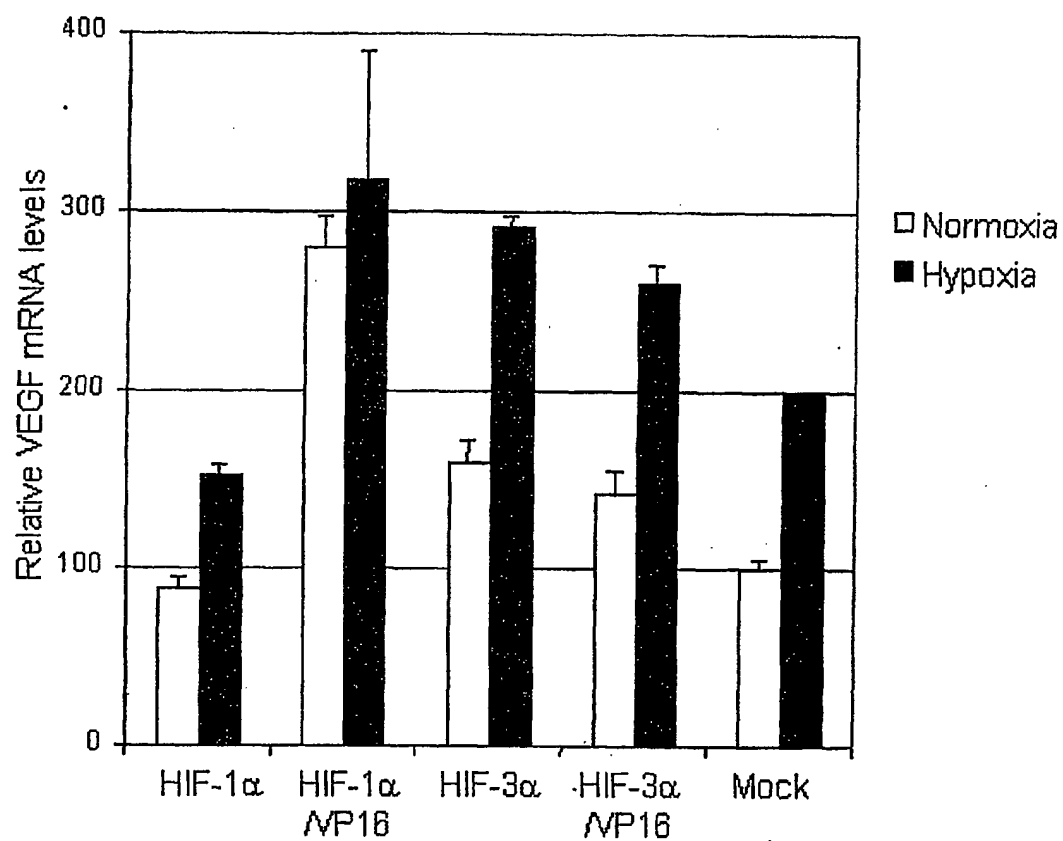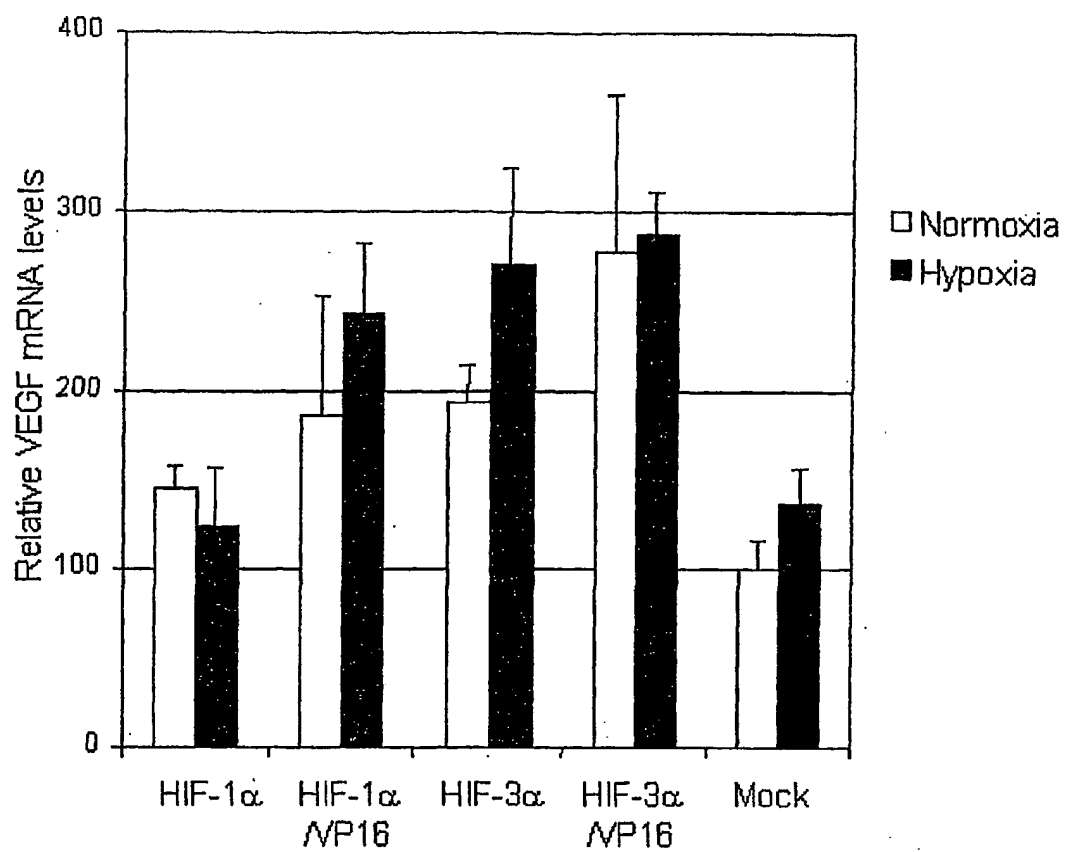
Figure 1

Figure 2A

Figure 2B
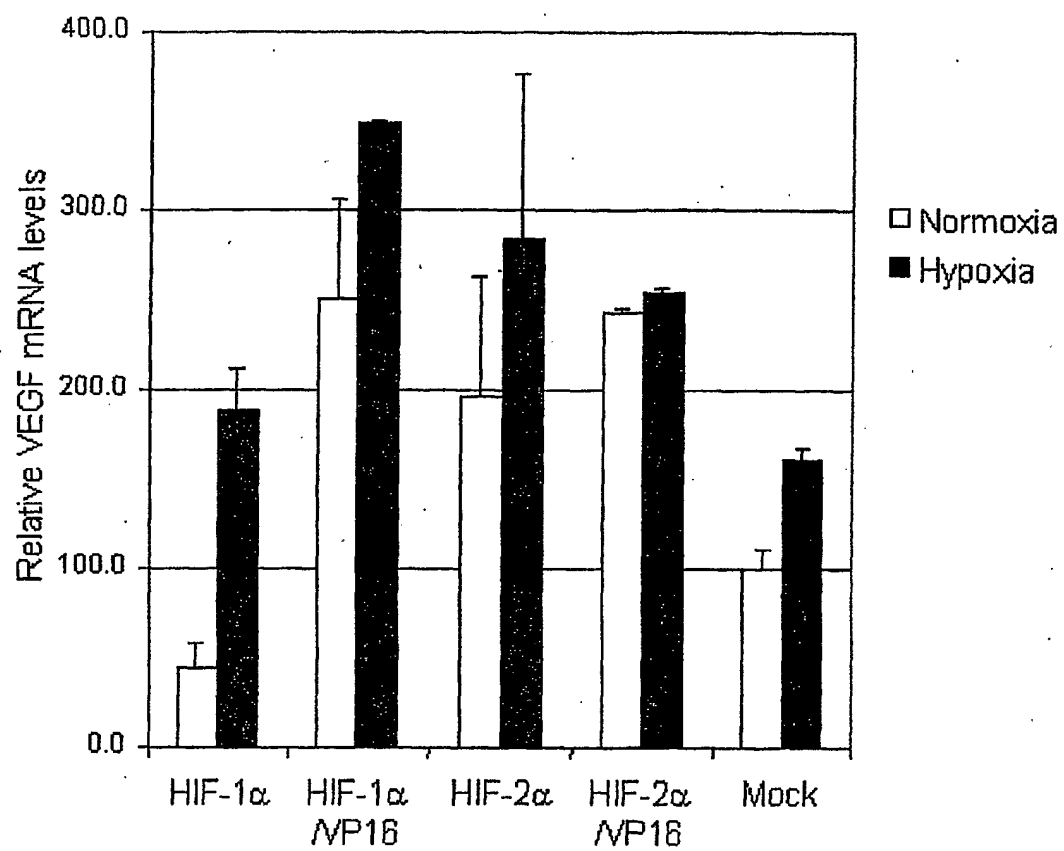
Figure 3A
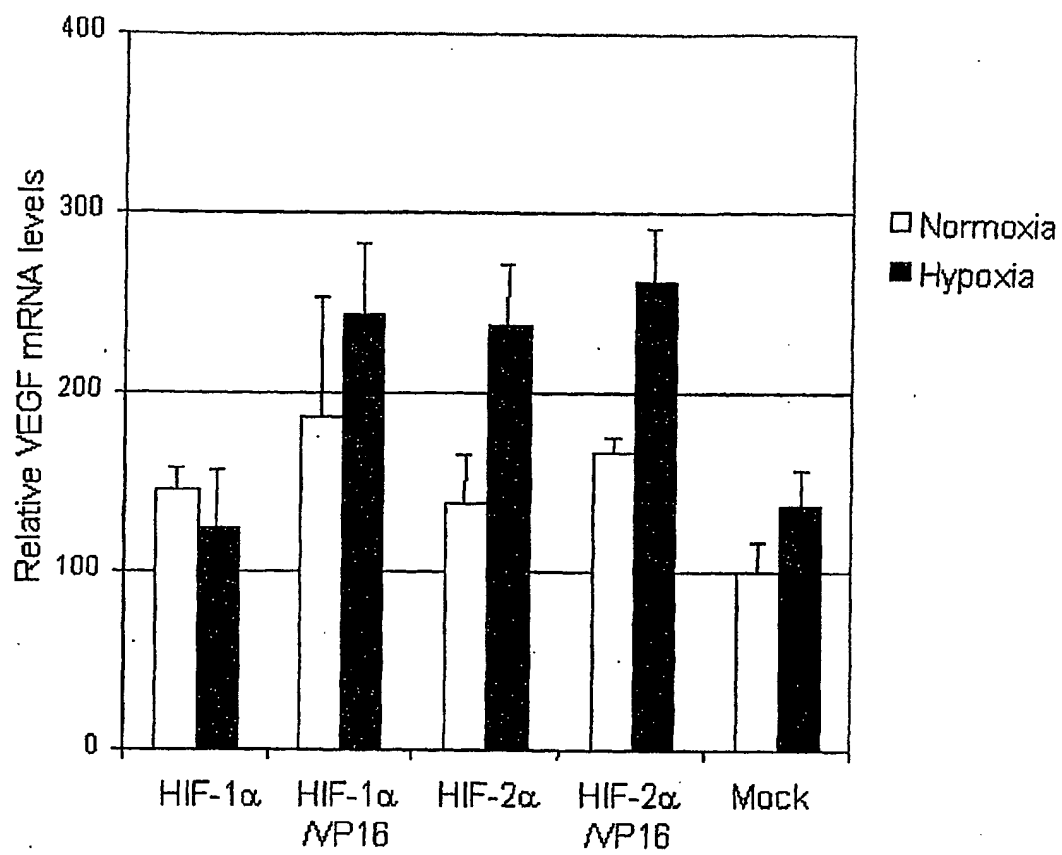
Figure 3B
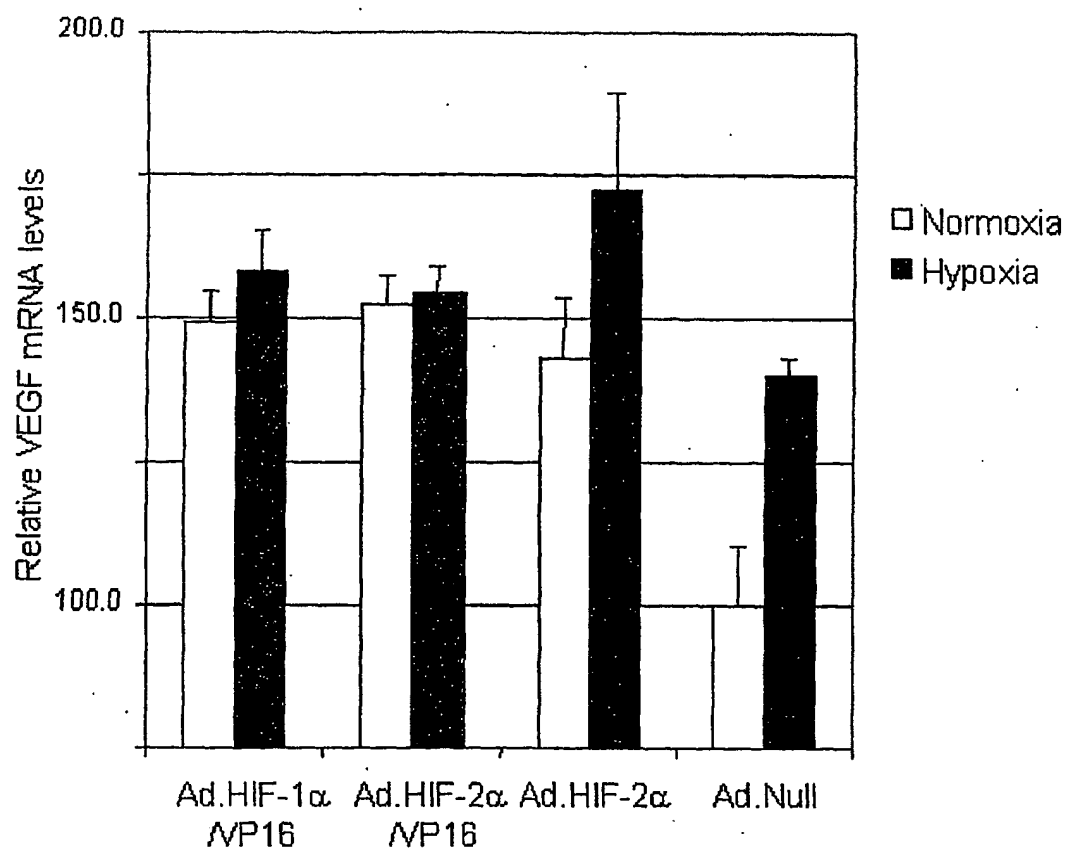
Figure 3C
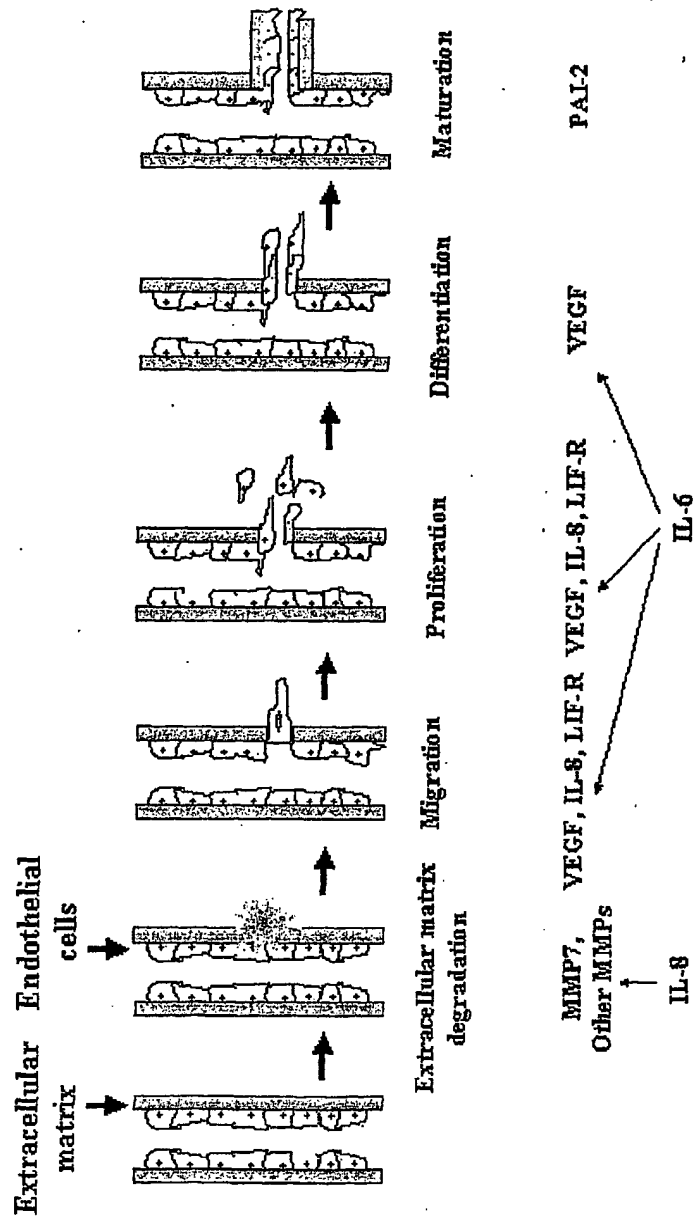
Figure 4
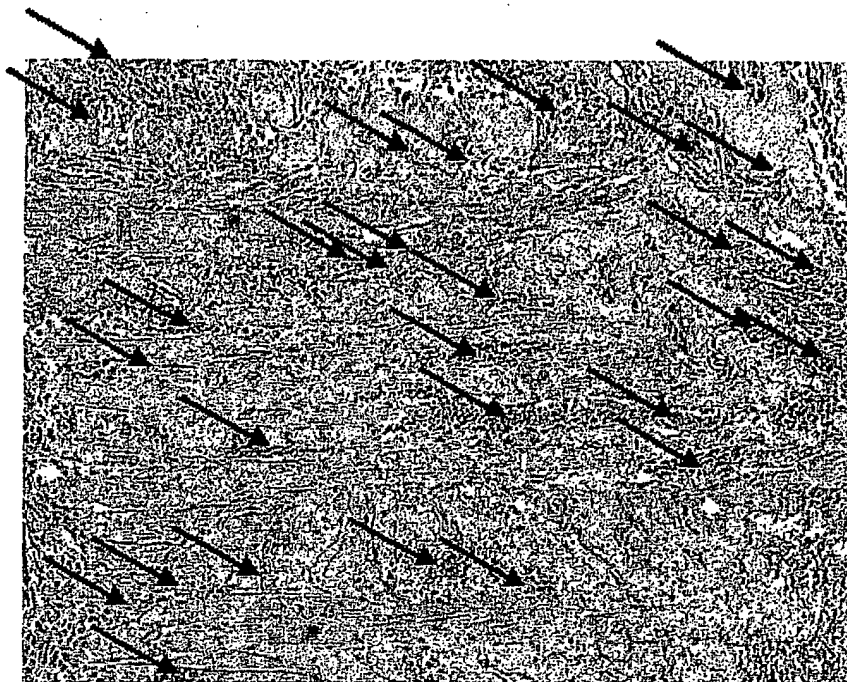
Figure 5A
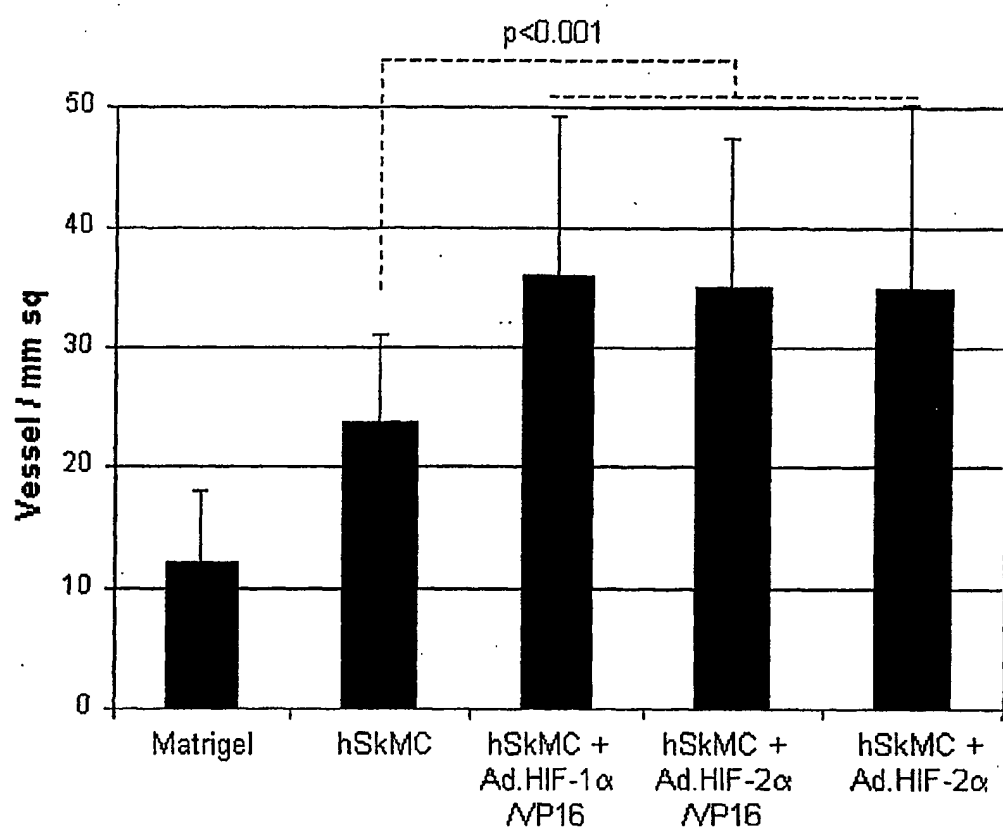
Figure 5B
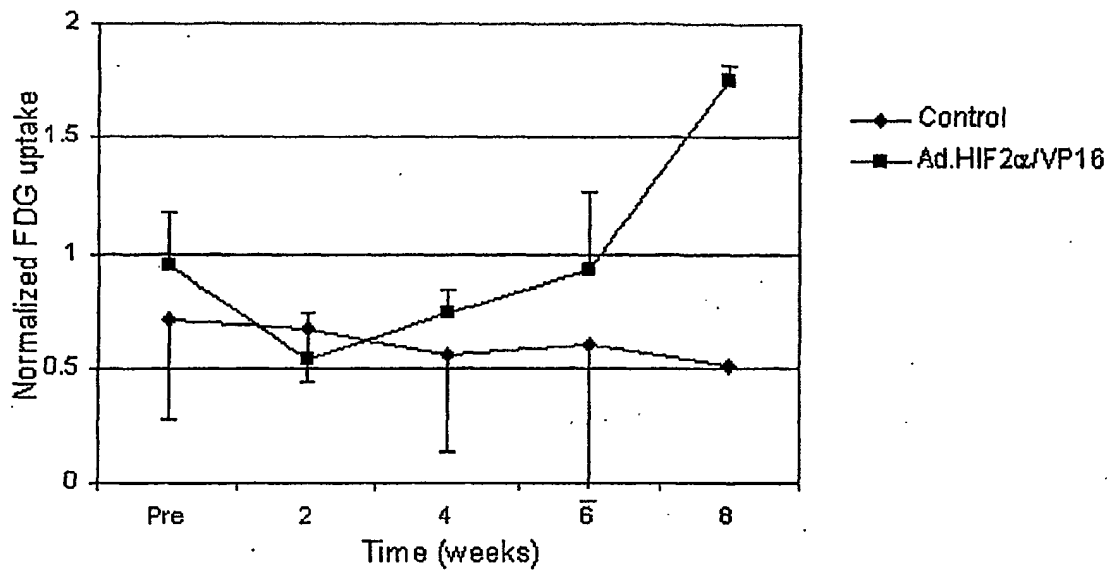
Figure 6A
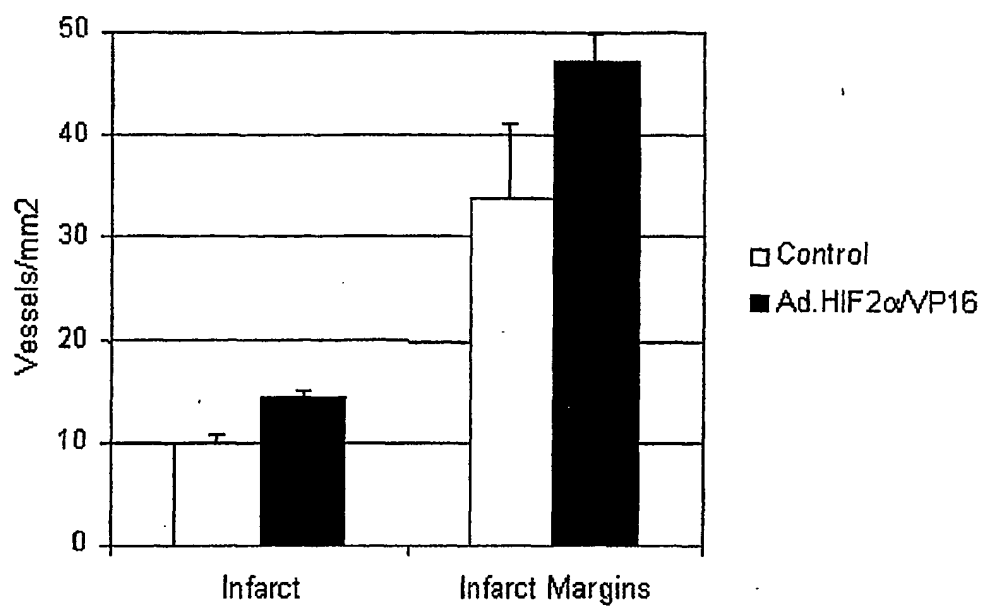
Figure 6B

SEQUENCE LISTING

<110> Angiogene Inc.

<120> HIPOXIA INDUCING FACTORS AND USES THEREOF FOR INDUCING ANGIOGENESIS
       AND IMPROVING MUSCULAR FUNCTIONS

<130> 000266-0008

<150> US 60/292,630
<151> 2001-05-22

<150> US60/354,529
<151> 2002-02-08

<160> 13

<170> PatentIn version 3.1

<210> 1
<211> 2223
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(2115)
<223>

<400> 1
atg gac agg tcg acc acg gag ctg cgc aag gaa aag tcc cgg gat gcg   48
Met Asp Arg Ser Thr Thr Glu Leu Arg Lys Glu Lys Ser Arg Asp Ala
1   5   10   15

gcc cgc agc cgg cgc agc cag gag acc gag gtg ctg tac cag ctg gct   96
Ala Arg Ser Arg Arg Ser Gln Glu Thr Glu Val Leu Tyr Gln Leu Ala
                20   25   30

cac acg ctg ccc ttc gcc cgc ggc gtc agc gcc cac ctg gac aag gcc   144
His Thr Leu Pro Phe Ala Arg Gly Val Ser Ala His Leu Asp Lys Ala
            35   40   45

tct atc atg cgc ctc acc atc agc tac ctg cgc atg cac cgc ctc tgc   192
Ser Ile Met Arg Leu Thr Ile Ser Tyr Leu Arg Met His Arg Leu Cys
        50   55   60

gcc gca ggg gag tgg aac cag gtg gga gca ggg gga gaa cca ctg gat   240
Ala Ala Gly Glu Trp Asn Gln Val Gly Ala Gly Gly Glu Pro Leu Asp
65   70   75   80

gcc tgc tac ctg aag gcc ctg gag ggc ttc gtc atg gtg ctc acc gcc   288
Ala Cys Tyr Leu Lys Ala Leu Glu Gly Phe Val Met Val Leu Thr Ala
                85   90   95

gag gga gac atg gct tac ctg tcg gag aat gtc agc aaa cac ctg ggc   336
Glu Gly Asp Met Ala Tyr Leu Ser Glu Asn Val Ser Lys His Leu Gly
                100   105   110

ctc agt cag ctg gag ctc att gga cac agc atc ttt gat ttc atc cac   384

- 2/10 -

```
Leu Ser Gln Leu Glu Leu Ile Gly His Ser Ile Phe Asp Phe Ile His
        115             120             125

ccc tgt gac caa gag gag ctt cag gac gcc ctg acc ccc caa cag acc    432
Pro Cys Asp Gln Glu Glu Leu Gln Asp Ala Leu Thr Pro Gln Gln Thr
        130             135             140

ctg tcc agg agg aag gtg gag gcc ccc acg gag cgg tgc ttc tcc ttg    480
Leu Ser Arg Arg Lys Val Glu Ala Pro Thr Glu Arg Cys Phe Ser Leu
145             150             155             160

cgc atg aag agt acg ctc acc agc cgc ggg cgc acc ctc aac ctc aag    528
Arg Met Lys Ser Thr Leu Thr Ser Arg Gly Arg Thr Leu Asn Leu Lys
                165             170             175

gcg gcc acc tgg aag gtg ctg aac tgc tct gga cat atg agg gcc tac    576
Ala Ala Thr Trp Lys Val Leu Asn Cys Ser Gly His Met Arg Ala Tyr
                180             185             190

aag cca cct gcg cag act tct cca gct ggg agc cct gac tca gag ccc    624
Lys Pro Pro Ala Gln Thr Ser Pro Ala Gly Ser Pro Asp Ser Glu Pro
            195             200             205

ccg ctg cag tgc ccg gtg ctc atc tgc gaa gcc atc ccc cac cca ggc    672
Pro Leu Gln Cys Pro Val Leu Ile Cys Glu Ala Ile Pro His Pro Gly
        210             215             220

agc ctg gag ccc cca ctg ggc cga ggg gcc ttc ctc agc cgc cac agc    720
Ser Leu Glu Pro Pro Leu Gly Arg Gly Ala Phe Leu Ser Arg His Ser
225             230             235             240

ctg gac atg aag ttc acc tac tgt gac gac agg att gca gaa gtg gct    768
Leu Asp Met Lys Phe Thr Tyr Cys Asp Asp Arg Ile Ala Glu Val Ala
                245             250             255

ggc tat agt ccc gat gac ctg atc ggc tgt tcc gcc tac gag tac atc    816
Gly Tyr Ser Pro Asp Asp Leu Ile Gly Cys Ser Ala Tyr Glu Tyr Ile
            260             265             270

cac gcg ctg gac tcc gac gcg gtc agc aag agc atc cac acc ttg ctg    864
His Ala Leu Asp Ser Asp Ala Val Ser Lys Ser Ile His Thr Leu Leu
            275             280             285

agc aag ggc cag gca gta aca ggg cag tat cgc ttc ctg gcc cgg agt    912
Ser Lys Gly Gln Ala Val Thr Gly Gln Tyr Arg Phe Leu Ala Arg Ser
        290             295             300

ggt ggc tac ctg tgg acc cag acc cag gcc aca gtg gtg tca ggg gga    960
Gly Gly Tyr Leu Trp Thr Gln Thr Gln Ala Thr Val Val Ser Gly Gly
305             310             315             320

cgg ggc ccc cag tcg gag agt atc gtc tgt gtc cat ttt tta atc agc   1008
Arg Gly Pro Gln Ser Glu Ser Ile Val Cys Val His Phe Leu Ile Ser
                325             330             335

cag gtg gaa gag acc gga gtg gtg ctg tcc ctg gag caa acg gag caa   1056
Gln Val Glu Glu Thr Gly Val Val Leu Ser Leu Glu Gln Thr Glu Gln
                340             345             350

cac tct cgc aga ccc att cag cgg ggc gcc ccc tct cag aag ggc acc   1104
His Ser Arg Arg Pro Ile Gln Arg Gly Ala Pro Ser Gln Lys Gly Thr
```

```
            355                       360                       365

cct aac cct ggg gac agc ctt gac acc cct ggc ccc cgg atc ctt gcc    1152
Pro Asn Pro Gly Asp Ser Leu Asp Thr Pro Gly Pro Arg Ile Leu Ala
    370               375               380

ttc ctg cac ccg cct tcc ctg agc gag gct gcc ctg gcc gct gac ccc    1200
Phe Leu His Pro Pro Ser Leu Ser Glu Ala Ala Leu Ala Ala Asp Pro
385               390               395               400

cgc cgt ttc tgc agc cct gac ctc cgt cgc ctc ctg gga ccc atc ctg    1248
Arg Arg Phe Cys Ser Pro Asp Leu Arg Arg Leu Leu Gly Pro Ile Leu
              405               410               415

gat ggg gct tca gta gca gcc act ccc agc acc ccg ctg gcc aca cgg    1296
Asp Gly Ala Ser Val Ala Ala Thr Pro Ser Thr Pro Leu Ala Thr Arg
              420               425               430

cac ccc caa agt cct ctt tcg gct gat ctc cca gat gaa cta cct gtg    1344
His Pro Gln Ser Pro Leu Ser Ala Asp Leu Pro Asp Glu Leu Pro Val
              435               440               445

ggc acc gag aat gtg cac aga ctc ttc acc tcc ggg aaa gac act gag    1392
Gly Thr Glu Asn Val His Arg Leu Phe Thr Ser Gly Lys Asp Thr Glu
    450               455               460

gca gtg gag aca gat tta gat ata gct cag atg agg aaa ctg aag ctc    1440
Ala Val Glu Thr Asp Leu Asp Ile Ala Gln Met Arg Lys Leu Lys Leu
465               470               475               480

aga ctg ttg acc aca ggc aca gaa ctc aga agt gat ggt gct ggg act    1488
Arg Leu Leu Thr Thr Gly Thr Glu Leu Arg Ser Asp Gly Ala Gly Thr
              485               490               495

tca gcc aag gtc cac cca agt cca agg ctc atc ctc tta cct ccc tcc    1536
Ser Ala Lys Val His Pro Ser Pro Arg Leu Ile Leu Leu Pro Pro Ser
              500               505               510

tgc cct ccg cag gat gct gat gct ctg gat ttg gag atg ctg gcc ccc    1584
Cys Pro Pro Gln Asp Ala Asp Ala Leu Asp Leu Glu Met Leu Ala Pro
              515               520               525

tac atc tcc atg gat gat gac ttc cag ctc aac gcc agc gag cag cta    1632
Tyr Ile Ser Met Asp Asp Asp Phe Gln Leu Asn Ala Ser Glu Gln Leu
    530               535               540

ccc agg gcc tac cac aga cct ctg ggg gct gtc ccc cgg ccc cgt gct    1680
Pro Arg Ala Tyr His Arg Pro Leu Gly Ala Val Pro Arg Pro Arg Ala
545               550               555               560

cgg agc ttc cat ggc ctg tca cct cca gcc ctt gag ccc tcc ctg cta    1728
Arg Ser Phe His Gly Leu Ser Pro Pro Ala Leu Glu Pro Ser Leu Leu
              565               570               575

ccc cgc tgg ggg agt gac ccc cgg ctg agc tgc tcc agc cct tcc aga    1776
Pro Arg Trp Gly Ser Asp Pro Arg Leu Ser Cys Ser Ser Pro Ser Arg
              580               585               590

ggg gac ccc tca gca tcc tct ccc atg gct ggg gct cgg aag agg acc    1824
Gly Asp Pro Ser Ala Ser Ser Pro Met Ala Gly Ala Arg Lys Arg Thr
              595               600               605
```

SUBSTITUTE SHEET (RULE 26)

```
ctg gcc cag agc tca gag gac gag gac gag gga gtg gag ctg ctg gga    1872
Leu Ala Gln Ser Ser Glu Asp Glu Asp Glu Gly Val Glu Leu Leu Gly
    610             615             620

gtg aga cct ccc aaa agg tcc ccc agc cca gaa cac gaa aac ttt ctg    1920
Val Arg Pro Pro Lys Arg Ser Pro Ser Pro Glu His Glu Asn Phe Leu
625             630             635             ·640

ctc ttt cct ctc agc ctg agt ttc ctt ctg aca gga gga cca gcc cca    1968
Leu Phe Pro Leu Ser Leu Ser Phe Leu Leu Thr Gly Gly Pro Ala Pro
                645             650             655

ggg agc ctg cag gac ccc agc acc cca ctc ctg aac ctg aat gag ccc    2016
Gly Ser Leu Gln Asp Pro Ser Thr Pro Leu Leu Asn Leu Asn Glu Pro
            660             665             670

ctg ggc ctg ggc ccc tca ctg ctc tct ccg tac tca gac gag gac act    2064
Leu Gly Leu Gly Pro Ser Leu Leu Ser Pro Tyr Ser Asp Glu Asp Thr
    675             680             685

acc cag ccc ggg ggc ccc ttc cag cca agg gca ggc tca gcc cag gct    2112
Thr Gln Pro Gly Gly Pro Phe Gln Pro Arg Ala Gly Ser Ala Gln Ala
    690             695             700             ⸜

gac tgagccggct cctctcccca tctgccttct cctcccccag aaaggacctc         2165
Asp
705

aaccacactc cacgccggca gccaacgcac aggaggtcct tgccttccgg caccaacg    2223
```

```
<210>  2
<211>  705
<212>  PRT
<213>  Homo sapiens

<400>  2

Met Asp Arg Ser Thr Thr Glu Leu Arg Lys Glu Lys Ser Arg Asp Ala
1               5               10              15

Ala Arg Ser Arg Arg Ser Gln Glu Thr Glu Val Leu Tyr Gln Leu Ala
            20              25              30

His Thr Leu Pro Phe Ala Arg Gly Val Ser Ala His Leu Asp Lys Ala
        35              40              45

Ser Ile Met Arg Leu Thr Ile Ser Tyr Leu Arg Met His Arg Leu Cys
    50              55              60

Ala Ala Gly Glu Trp Asn Gln Val Gly Ala Gly Gly Glu Pro Leu Asp
65              70              75              80

Ala Cys Tyr Leu Lys Ala Leu Glu Gly Phe Val Met Val Leu Thr Ala
            85              90              95
```

```
Glu Gly Asp Met Ala Tyr Leu Ser Glu Asn Val Ser Lys His Leu Gly
            100             105             110

Leu Ser Gln Leu Glu Leu Ile Gly His Ser Ile Phe Asp Phe Ile His
        115             120             125

Pro Cys Asp Gln Glu Glu Leu Gln Asp Ala Leu Thr Pro Gln Gln Thr
    130             135             140

Leu Ser Arg Arg Lys Val Glu Ala Pro Thr Glu Arg Cys Phe Ser Leu
145             150             155             160

Arg Met Lys Ser Thr Leu Thr Ser Arg Gly Arg Thr Leu Asn Leu Lys
            165             170             175

Ala Ala Thr Trp Lys Val Leu Asn Cys Ser Gly His Met Arg Ala Tyr
            180             185             190

Lys Pro Pro Ala Gln Thr Ser Pro Ala Gly Ser Pro Asp Ser Glu Pro
    195             200             205

Pro Leu Gln Cys Pro Val Leu Ile Cys Glu Ala Ile Pro His Pro Gly
    210             215             220

Ser Leu Glu Pro Pro Leu Gly Arg Gly Ala Phe Leu Ser Arg His Ser
225             230             235             240

Leu Asp Met Lys Phe Thr Tyr Cys Asp Asp Arg Ile Ala Glu Val Ala
            245             250             255

Gly Tyr Ser Pro Asp Asp Leu Ile Gly Cys Ser Ala Tyr Glu Tyr Ile
            260             265             270

His Ala Leu Asp Ser Asp Ala Val Ser Lys Ser Ile His Thr Leu Leu
            275             280             285

Ser Lys Gly Gln Ala Val Thr Gly Gln Tyr Arg Phe Leu Ala Arg Ser
    290             295             300

Gly Gly Tyr Leu Trp Thr Gln Thr Gln Ala Thr Val Val Ser Gly Gly
305             310             315             320

Arg Gly Pro Gln Ser Glu Ser Ile Val Cys Val His Phe Leu Ile Ser
            325             330             335
```

Gln Val Glu Glu Thr Gly Val Val Leu Ser Leu Glu Gln Thr Glu Gln
        340   345   350

His Ser Arg Arg Pro Ile Gln Arg Gly Ala Pro Ser Gln Lys Gly Thr
        355   360   365

Pro Asn Pro Gly Asp Ser Leu Asp Thr Pro Gly Pro Arg Ile Leu Ala
    370   375   380

Phe Leu His Pro Pro Ser Leu Ser Glu Ala Ala Leu Ala Ala Asp Pro
385   390   395   400

Arg Arg Phe Cys Ser Pro Asp Leu Arg Arg Leu Leu Gly Pro Ile Leu
            405   410   415

Asp Gly Ala Ser Val Ala Ala Thr Pro Ser Thr Pro Leu Ala Thr Arg
            420   425   430

His Pro Gln Ser Pro Leu Ser Ala Asp Leu Pro Asp Glu Leu Pro Val
        435   440   445

Gly Thr Glu Asn Val His Arg Leu Phe Thr Ser Gly Lys Asp Thr Glu
    450   455   460

Ala Val Glu Thr Asp Leu Asp Ile Ala Gln Met Arg Lys Leu Lys Leu
465   470   475   480

Arg Leu Leu Thr Thr Gly Thr Glu Leu Arg Ser Asp Gly Ala Gly Thr
            485   490   495

Ser Ala Lys Val His Pro Ser Pro Arg Leu Ile Leu Leu Pro Pro Ser
        500   505   510

Cys Pro Pro Gln Asp Ala Asp Ala Leu Asp Leu Glu Met Leu Ala Pro
        515   520   525

Tyr Ile Ser Met Asp Asp Asp Phe Gln Leu Asn Ala Ser Glu Gln Leu
    530   535   540

Pro Arg Ala Tyr His Arg Pro Leu Gly Ala Val Pro Arg Pro Arg Ala
545   550   555   560

Arg Ser Phe His Gly Leu Ser Pro Pro Ala Leu Glu Pro Ser Leu Leu
            565   570   575

SUBSTITUTE SHEET (RULE 26)

```
Pro Arg Trp Gly Ser Asp Pro Arg Leu Ser Cys Ser Ser Pro Ser Arg
        580               585      ..   .     590

Gly Asp Pro Ser Ala Ser Ser Pro Met Ala Gly Ala Arg Lys Arg Thr
    595               600               605

Leu Ala Gln Ser Ser Glu Asp Glu Asp Glu Gly Val Glu Leu Leu Gly
    610               615               620

Val Arg Pro Pro Lys Arg Ser Pro Ser Pro Glu His Glu Asn Phe Leu
625               630               635               640

Leu Phe Pro Leu Ser Leu Ser Phe Leu Leu Thr Gly Gly Pro Ala Pro
            645               650               655

Gly Ser Leu Gln Asp Pro Ser Thr Pro Leu Leu Asn Leu Asn Glu Pro
        660               665               670

Leu Gly Leu Gly Pro Ser Leu Leu Ser Pro Tyr Ser Asp Glu Asp Thr
    675               680               685

Thr Gln Pro Gly Gly Pro Phe Gln Pro Arg Ala Gly Ser Ala Gln Ala
    690               695               700

Asp
705
```

```
<210>  3
<211>  2115
<212>  DNA
<213>  Homo sapiens

<400>  3
atggacaggt cgaccacgga gctgcgcaag gaaaagtccc gggatgcggc ccgcagccgg      60

cgcagccagg agaccgaggt gctgtaccag ctggctcaca cgctgccctt cgcccgcggc     120

gtcagcgccc acctggacaa ggcctctatc atgcgcctca ccatcagcta cctgcgcatg     180

caccgcctct cgccgcagg ggagtggaac caggtgggag cagggggaga accactggat     240

gcctgctacc tgaaggccct ggagggcttc gtcatggtgc tcaccgccga gggagacatg     300

gcttacctgt cggagaatgt cagcaaacac ctgggcctca gtcagctgga gctcattgga     360

cacagcatct ttgatttcat ccacccctgt gaccaagagg agcttcagga cgccctgacc     420

ccccaacaga ccctgtccag gaggaaggtg gaggccccca cggagcggtg cttctccttg     480

cgcatgaaga gtacgctcac cagccgcggg cgcaccctca acctcaaggc ggccacctgg     540

aaggtgctga actgctctgg acatatgagg gcctacaagc cacctgcgca gacttctcca     600
```

```
<400>  9
cagctggagc tcattggaca cagcatc                                          27


<210>  10
<211>  24
<212>  DNA
<213>  artificial sequence

<220>
<223>  Sequence is completely synthesized

<400>  10
ccccatcctg tgcgttggct gccg                                             24


<210>  11
<211>  28
<212>  DNA
<213>  artificial sequence

<220>
<223>  Sequence is completely synthesized

<400>  11
ggagtcagct taagctgaat gggtctgc                                         28


<210>  12
<211>  26
<212>  DNA
<213>  artificial sequence

<220>
<223>  Sequence is completely synthesized

<400>  12
ccggaattct ctacctccac catgcc                                           26


<210>  13
<211>  28
<212>  DNA
<213>  artificial sequence

<220>
<223>  Sequence is completely synthesized

<400>  13
ccggaattcc tcagtgggca cacactcc                                         28
```